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Increasing Axonal Arborization Size of Dopamine Neurons to Produce a Better Mouse Model of Parkinson's Disease

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RÉSUMÉ

Dans la maladie de Parkinson, les neurones dopaminergiques (DA) de la substance noire compacte (SNc) sont particulièrement vulnérables dû à leurs très grande taille de l'arborisation axonale, des besoins énergétiques très élevés et du stress oxydatif chroniquement élevé associés à ce phénotype. Étrangement, les modèles génétiques murins de la maladie de Parkinson ne montrent pas de dégénérescence spontanée des neurones DA. Notre hypothèse principale est que suite à une lésion partielle des neurones DA de la SNc, les neurones survivants montreront un bourgeonnement axonal compensatoire, ce qui résultera, chez la souris adulte, en une population de neurones DA dotée d'une arborisation axonale beaucoup plus grande et d'une vulnérabilité basale accrue. Une lésion unilatérale d'approximativement 50% des neurones DA était induite dans la SNc par une injection unilatérale de 6-hydroxydopamine (6-OHDA) chez des souris de 5 jours des deux sexes. Les souris ont alors ensuite évaluées à l'âge de 3 mois. Dans une première étape, nous avons quantifié la taille de l'arborisation axonale des neurones DA en infectant une sous-population de ces neurones avec un virus AAV-EYFP. Dans une deuxième étape, la vulnérabilité des neurones DA était évaluée à l'âge de P135, en injectant les souris avec une virus d'alpha-synucléin à P90. Nos résultats montrent qu'à la suite d'une lésion partielle des neurones DA de la SNC, les neurones survivants se compensent en augmentant leur taille d'arborisation axonale par environ 2 fois. Cette compensation se traduit par environ 3-fois l'augmentation de la vulnérabilité de ces neurones lorsqu'ils sont exposés à un stress secondaire.

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Mots-clés : Maladie de Parkinson, dopamine, modèle animal, néonatal, 6-hydroxydopamine, arborisation axonale, croissance compensatoire, vulnérabilité, substance noire, aire tegmentaire ventrale

ABSTRACT

In Parkinson's disease (PD), dopaminergic neurons of the substantia nigra pars compacta (SNc) are one of the key subsets of neurons particularly vulnerable to degeneration. Research has only started to elucidate some of the potential causes of this selective vulnerability, but the exceptionally large and complex axonal arborization of these neurons appears to play an important role due to its impact of cellular bioenergetics and oxidative stress. Unfortunately, genetic mouse models of PD have until now not been able to replicate the spontaneous loss over time of SNc dopaminergic (DA) neurons. This could be due in part to the fact that DA neurons in mice do not have an axonal arborization as developed as that of DA neurons in the human brain. We hypothesized that manipulations which increase axonal arborization size in SNc dopamine neurons in mice will increase their vulnerability to cellular stress, resulting in a greater risk of cell death. Our objective was to force SNc neurons to develop a larger than normal axonal arborization, thereby increasing their energy expenditures and vulnerability. An approximate 60% lesion of dopaminergic neurons was induced in the SNc through unilateral injection of 6-hydroxydopamine (6-OHDA) in male and female neonatal mice. Axonal arborization size was quantified at P90 using a conditional AAV-EYFP virus and confocal microscopy. In the second phase of the project, we examined the vulnerability of SNc DA at P135 neurons to subsequent viral overexpression of alpha-synuclein. Our results show that a partial lesion of SNc dopamine neurons in neonatal mice induced an approximate 2-fold increase in compensatory sprouting of surviving neurons and, by consequence, greatly increased the density of their axonal projections to the striatum. This compensatory process increased the vulnerability of the surviving SNc DA neurons to alpha-synuclein toxicity by nearly 3-fold. These findings are compatible with our initial hypothesis and suggest that producing mice that have DA neurons with larger axonal arborizations may facilitate the development of better mouse models of PD.

Keywords: Parkinson's disease, dopamine, animal model, neonatal, 6-hydroxydopamine, axonal arborization, compensatory sprouting, vulnerability, Substantia Nigra, Ventral Tegmental Area

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LIST OF ACRONYMS

5-HT: Serotonin

6-OHDA: 6-hydroxydopamine

AA: Ascorbic Acid

AADC: aromatic-L-amino-acid decarboxylase

AAV EYFP: Adeno-associated Virus Enhanced Yellow Fluorescent Protein

Acb: Nucleus Accumbens

ACh: Acetylcholine

AMPA: alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AS: Alpha-synuclein

ATP: Adenosine triphosphate

CB: calbindin

Cl⁻: Chloride

CN: Caudate nucleus

CNS: Central nervous system

COMT: Catechol-O-methyl transferase

CPu: Caudate putamen

CSF: Cerebrospinal fluid

DA: Dopamine

DAB: 3,3'-diaminobenzidine

DAT: dopamine transporter

DBS: Deep brain stimulation

DIC: Dat-Ires-Cre

DJ-1: Protein deglycase DJ-1

DNA: Deoxyribonucleic acid

dSTR: dorsal striatum

ECF: Extracellular fluid

ER: Endoplasmic reticulum

GABA: gamma-amino butyric acid

GAD: Glutamate decarboxylase
GDNF: Glial cell-derived neurotrophic factor
GFP: Green fluorescent protein
GPe: external segment of the Globus pallidus
GPi: internal segment of the Globus Pallidus
hNSCs: Human neural stem cells
iGluR: ionotropic glutamate receptor
KO: Knockout
L: Lesioned
LB: Lewy body
LC: Locus coeruleus
L-DOPA: Levodopa
LRRK2: Leucine-rich repeat kinase 2
LTD: Long-term depression
LTP: Long-term potentiation
mAChR: muscarinic acetylcholine receptor
MAO-B: Monoamine oxidase B
MFB: Medial forebrain bundle
mGluR: metabotropic glutamate receptor
MPTP: 1-methyl-1,2,3,4-tetrahydropyridine
mRNA: messenger RNA
MSN: Medium spiny neurons
mtDNA: Mitochondrial DNA
NA: Noradrenaline
Na⁺: Sodium
nAChR: nicotinic acetylcholine receptor
NMDA: *N*-methyl-D-aspartate
NSCs: Neural stem cells
NSF: N-ethylmaleimide-sensitive factor

NT: Neurotransmitter

OXPHOS: oxidative phosphorylation

PBS: Phosphate buffered saline

PD: Parkinson's Disease

PFA: Paraformaldehyde

PINK-1: PTEN-induced putative kinase 1

Put: Putamen

RBD: REM sleep behaviour disorder

ROS: Reactive oxygen species

RRF : retrorubral field

SN: Substantia nigra

SN: Substantia nigra

SNARE: Soluble NSF attachment protein receptor

SNC: Substantia nigra pars compacta

SNCA: alpha-synuclein gene

SNC-d: dorsal tier of the SNC

SNC-v: ventral tier of the SNC

SNr: Substantia nigra pars reticulata

SPNs: Spiny projection neurons

STh: subthalamus

STN: Subthalamic nucleus

TH: Tyrosine hydroxylase

UCHL1: Ubiquitin carboxy-terminal hydrolase L1

UL: Unlesioned

VGAT: Vesicular glutamate transporter

VGLUT2: Vesicular glutamate transporter-2

VMAT2: Vesicular monoamine transporter 2

vSTR: ventral striatum

VT: Volume transmission

VTA: Ventral tegmental area

WT: Wild-type

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INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease, holding a lifetime risk of 4-5% (Mullin & Schapira, 2013). The number of cases of PD is expected to double by 2050, and current therapies are only symptom-treating and do not stop the progression of the disease. Characterized by a progressive dopaminergic denervation of the striatum, PD is caused by a gradual loss of dopamine (DA) neurons in the substantia nigra (SN). The dopaminergic deficit resulting from the progressive cell loss in PD results in significant motor impairments, including: resting tremor, rigidity, bradykinesia and postural instability (Mullin & Schapira, 2013; Iancu et al., 2005). Often, these motor disturbances are preceded by other, subtler non-motor symptoms such as anosmia, sleep disturbances, pain and other sensory abnormalities and impaired cognition (Le, Sayana & Jankovic, 2014; Mullin & Schapira, 2013).

The causes of PD are varied and complex and have been a primary focus of the scientific community in recent time. Whereas genetic and environmental factors have been shown to contribute to the development of PD, the specific mechanisms resulting in the selective degeneration of SN DA neurons continue to be elucidated. To date however, there is evidence to support several factors contributing to this degeneration, including: protein mishandling, oxidative stress, increased energetic demands and mitochondrial dysfunction (Surmeier et al., 2011; Berman & Hastings, 1999; Van Laar et al., 2008; Bolam & Pissadaki, 2012).

Contrary to prior beliefs, PD is now understood to be a dynamic and widespread disease. Whereas dopaminergic cell death in the SN is responsible for most of the motor symptoms observed in PD, cell death is not confined to this region. Lewy body (LB) pathology and cell death have been suggested to begin in the brain stem and undergo a slow progression toward the forebrain. The cardinal motor symptoms of PD are expressed when this pathology reaches the midbrain (Braak, 2003; Bolam & Pissadaki, 2012). It has been estimated that the primary symptoms of PD only become observable when the density of dopaminergic terminals in the striatum decreases by approximately 70% below normal levels, associated with the loss of 80-90% of DA cell bodies (Hefti, Melamed & Wurtman, 1980; Tadaiesky et al., 2008).

Recent evidence has started to accumulate regarding potential compensatory mechanisms which help explain this delay of symptom manifestation. To date, many various short-term and long-term mechanisms have been discovered, one of which involves compensatory sprouting. It is currently believed that the gradual cell death of SNC DA neurons triggers a compensatory axonal sprouting response (Finkelstein et al., 2000; Bolam & Pissadaki, 2012). In the context of such compensatory sprouting, the axonal arborization size of the surviving DA neurons is likely to be increased, leading to reinnervation of the partially denervated striatum.

PD does not spontaneously develop in rodents as it does in humans. Whereas their shorter lifespan is considered a contributing factor, a more recent theory is that dopaminergic SN neurons in humans are so much larger and more complex than those of rodents, and the bioenergetic needs are so much greater, that human SNC DA neurons are much more vulnerable to environmental and genetic stressors than those of rodents (Oorschot, 1996; Yin et al., 2009; Bolam & Pissadaki, 2012). Based on work carried out in culture, it has been hypothesized that this increase in axonal arborization size contributes to the increased vulnerability of SNC DA neurons through elevated bioenergetic demands, increased oxidative stress, and mitochondrial dysfunction (Pacelli et al., 2015).

Current neurotoxic and genetic models of PD fail to take this increased size and vulnerability into consideration. Therefore, the hypotheses for the current thesis project were two-fold: first, that a partial unilateral lesion of the SNC of neonatal mice will induce compensatory sprouting, resulting in adult mice with fewer DA neurons and much larger axonal arborizations, more representative of what is occurring in the human pathology. Second, that this larger arborization size would increase the vulnerability of these neurons upon exposure to secondary stressors. It is expected that this new model of PD would be more representative of the human pathology and would give us the opportunity to further our understanding of the mechanisms involved in the pathological progression of this disease, as well as the ability to identify more effective treatment strategies than are currently available.

THE DOPAMINERGIC SYSTEM

The dopaminergic system, discovered roughly 50 years ago, has become one of the most extensively studied neurotransmitter systems in the brain (Greer & Williams, 1963; Barbeau et al., 1963; O'Reilly, Loncin & Cooksey, 1965; Björklund & Dunnett, 2007). Whereas impressive strides have been made in this field since that time, the dynamic and adaptive properties of DA neurotransmission mean there is much yet to be discovered and explained. The separation of the midbrain DA projections into functionally and anatomically distinct components proposed decades ago remain valid today, however, we have come to understand that these systems are a lot more complex and intertwined than previously believed. A solid understanding of the dopaminergic system, its organization, and its role in essential functions such as motor control, and emotional and cognitive processes, is fundamental to our understanding of neurodegenerative diseases such as PD. Amongst all DA terminal regions of the brain, the striatum, divided into the caudate putamen (dorsal striatum) and nucleus accumbens (ventral striatum), is of important focus in PD research. This is due to its involvement in the aforementioned essential functions, the disruption of which results in some of the cardinal motor symptoms of this disease.

NEUROMODULATORY MECHANISMS

DOPAMINE

Dopamine is produced when L-tyrosine is converted to L-DOPA (3,4-dihydroxyphenylalanine) by the enzyme tyrosine hydroxylase (TH), which is subsequently converted into DA by the enzyme aromatic-L-amino-acid decarboxylase (AADC). Once DA has either been synthesized in the cytoplasm of DA neurons or undergone cellular reuptake by the dopamine transporter (DAT), it is then packaged into vesicles by vesicular monoamine transporter 2 (VMAT2) and stored until its release (Morales & Margolis, 2017). Dopamine neither excites nor inhibits its target cells and is therefore often considered more of a neuromodulator than a neurotransmitter (NT). Excitability of GABAergic and cholinergic

interneurons and striatal MSNs is modulated by a dense innervation of mesencephalic dopaminergic axons. Each cell type has a differential class and combination of DA receptor expression, which affect the characteristics of dopaminergic modulation (Do et al., 2013). Synapsing primarily with GABA and glutamate neurons, DA also modulates the efficacy of signal transmission mediated by other neurotransmitters. Its effect is exerted through two distinct mechanisms: the phasic synaptic mode of dopaminergic signal transmission, and the tonic-nonsynaptic mode of dopaminergic transmission.

In the phasic synaptic mode of transmission, the sensitivity of the response of DA-receptive neurons to NT stimulation is altered by DA. The neuronal function modulated by DA is dependent on the DA-receptor subtype that is activated on the postsynaptic cell. Consequently, excitatory neurotransmission can be either facilitated or inhibited depending on the type of DA receptor subtype activated. For example, D1-type receptor activation enhances glutamatergic excitatory effects upon its binding to NMDA receptors, whereas activation of the D2 receptor results in an inhibition of glutamatergic effects upon its binding to the AMPA receptor (Cepeda, Buchwald & Levine, 1993; Di Chiara, 1997).

In the tonic-nonsynaptic mode of dopaminergic transmission, DA can modulate the neurotransmitter release that is induced by cellular excitation. Both dopaminergic and nondopaminergic cells possess extrasynaptic DA receptors which are activated upon DA release into the synaptic cleft. Activation of D1 and D2 extrasynaptic receptors can modulate either the release of DA itself, or the release of other neurotransmitters (i.e. acetylcholine, glutamate and GABA) by nondopaminergic neurons. Release of these neurotransmitters can be either enhanced or inhibited by activation of D1 and D2 extrasynaptic receptors, respectively (Starke, 1981; Chesselet, 1984; Di Chiara, 1997).

DA receptors are classified into two families: the D1-like family, which include receptor subtypes D1 and D5; and the D2-like family, which include subtypes D2, D3 and D4. These five G-protein coupled receptor subtypes mediate the diverse physiological actions of DA. D1-like receptors are coupled to the Gs (stimulatory) protein that activates adenyl cyclase, whereas the other receptors are coupled to Gi (inhibitory) proteins that inhibit adenyl cyclase and

activate potassium (K⁺) channels (Missale et al., 1998; Carrion et al., 2010). DA receptors have a rather heterogeneous pattern of distribution: D1-like receptor concentration is relatively higher than D2 in the prefrontal cortex, whereas D2-like receptor concentration is higher in the caudate nucleus, putamen and nucleus accumbens. Important to note is that whereas D1 and D2-like receptors exert opposing effects, they often exhibit a synergistic relationship when more complex outputs are considered (Sesack et al., 2003; Carrion et al., 2010).

DA receptors in the striatum are not distributed homogeneously among dopaminergic cells, therefore, the flow of information can be modulated by DA across the striatum in two ways: by stimulating either D1-family receptors or D2-family receptors via the tonic-nonsynaptic mechanism of action. Stimulation of the D1 family of receptors results in enhanced NT release and could allow DA to facilitate the transmission of information from the striatum to other brain areas. Stimulation of the D2-family receptors however, reduces NT release, thereby allowing DA to reduce unnecessary surrounding signals and enhancing the transmission of information across the striatum. (Cepeda, Buchwald & Levine, 1993; Martin & Waszczak, 1994; Di Chiara, 1997)

SEROTONIN

Whereas loss of nigrostriatal DA neurons is the defining feature of PD, increasing evidence indicates that degeneration of serotonin (5-HT) and norepinephrine neurons contribute to some clinically significant non-motor symptoms of PD. Serotonin-related alterations have also been shown to play a significant role in emotional, cognitive and motor-related PD symptoms (Carter & Pycock, 1979; Meyer et al., 2004; Brichta, Greengard & Flajolet, 2013). These neurons have been shown to have dynamic compensatory properties in response to DA depletion (Berger & Glowinski, 1978; Arai et al., 1994; Maeda et al., 2003).

5-HT neurons are located primarily in the raphe nuclei of the brainstem and provide 5-HT innervation to the entire brain, but particularly to structures of the basal ganglia. PD patients can present with serotonergic depletion as high as 85% in regions such as the SN, striatum, hypothalamus and thalamus (Kish et al., 2008; Huot, Fox & Brotchie, 2011). To date, 14 5-HT receptor subtypes have been identified, 13 of which are metabotropic and one ionotropic

(Nichols & Nichols, 2008; Huot, Fox & Brotchie, 2011). These receptor subtypes are implicated in various functions and are heterogeneously distributed throughout the brain. They also have various roles in modulating glutamatergic, serotonergic and dopaminergic neurotransmission in the basal ganglia.

NOREPINEPHRINE

PD is characterized not only by disrupted function of the SNC and basal ganglia networks, but also of cortical networks, particularly the primary motor cortex (Guo et al., 2015). Postmortem studies have revealed degeneration in the locus coeruleus (LC), which is responsible for supplying the cortex with noradrenaline (NA). The cerebellar cortex, thalamus and motor cortex have all been reported to have reduced levels of NA (Scatton et al., 1983; Sommerauer et al., 2018; Pifl, Kish & Hornykiewicz, 2012). Braak staging of PD reports the LC as being affected by alpha-synuclein aggregates in stage 2, which precedes and could surpass SNC involvement at a later timepoint in the disease progression. It is believed that this NA pathology is, in part, responsible for the mood, postural instability and gait abnormalities observed in PD (Pifl, Kish & Hornykiewicz, 2012; Brichta, Greengard & Flajolet, 2013). Some studies have shown that deficiencies in NA innervation can exacerbate dopaminergic cell loss in some neurotoxic models of PD (Mavridis et al., 1991; Fornai et al., 1996).

THE NEUROMOLECULAR MECHANISMS OF NEUROTRANSMITTERS

GLUTAMATE

Glutamate is an excitatory neurotransmitter that functions to activate the postsynaptic cell. Glutamate is important in regulating DAs modulatory functions. Glutaminase is the enzyme that produces glutamate from glutamine. Vesicular glutamate transporter-2 (VGLUT2) is the primary transporter expressed in glutamatergic neurons of the A10 area. The effect of the NT is transmitted through two types of receptors: ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs). Both receptors are widely expressed in various brain regions, including the basal ganglia. The way they mediate synaptic transmission depends

on the type of receptor activated: fast synaptic transmission and slow synaptic transmission is the result of iGluRs and mGluRs respectively (Brichta, Greengard & Flajolet, 2013). mGluRs can be located either presynaptically, postsynaptically, or both. They also have the ability to mediate both excitatory and inhibitor effects. Hyperactivation of glutamatergic projections in certain areas (STN and PPN) have implicated their involvement in the control of posture and gait in PD.

GAMMA-AMINOBUTYRIC ACID (GABA)

Gamma-aminobutyric acid (GABA) is an inhibitory neurotransmitter which depresses the activity of the postsynaptic cell. It is synthesized from glutamate by the enzyme glutamate decarboxylase (GAD1 or GAD2) and accumulated into vesicles by the vesicular GABA transporter (VGAT). Ionotropic GABA_A and metabotropic GABA_B receptors are the primary inhibitory receptors in the human basal ganglia (Waldvogel et al., 1999, 2004). GABA_A receptors are the most widespread inhibitory receptors in the CNS and have a variety of classes, depending on subunit formation. These receptors are localized on postsynaptic membranes of inhibitory synapses and facilitate fast-response, inhibitory neurotransmission.

GABAergic spiny projection neurons (SPNs) are one of the principal neurons in the striatum and can constitute up to 90% of the striatal neuron population. They are also one of the major targets of DA innervation. GABAergic SPNs can be divided into two populations: the direct pathway SPNs and the indirect pathway SPNs (Gerfen & Surmeier, 2011; Brichta Greengard & Flajolet, 2013). Each of these pathways exhibit a differential expression of DA receptors. D1 receptors and D2 receptors are selectively expressed by direct and indirect pathway SPNs respectively. Signaling alterations in these pathways change the output of the basal ganglia, which results in some of the motor symptoms of PD (Gerfen & Surmeier, 2011; Brichta Greengard & Flajolet, 2013). Research studying advanced PD has shown that GABAergic SPNs in this phase of the disease are characterized by truncated dendrites and a reduced number of spines.

ACETYLCHOLINE

Acetylcholine (ACh) is another neuromodulatory system affected in PD. Cholinergic degeneration has been shown to contribute to cognitive impairments, psychosis, gait impairments and REM-sleep disturbances. These ACh interneurons are large, aspiny and possess a dense widespread innervation of dendritic arbors in the striatum where they are tonically active (Bolam, 1984). There are two types of ACh receptors: nicotinic and muscarinic. Nicotinic ACh receptors (nAChR) are able to locally regulate DA release and enhance DA release (Zhou et al., 2001; Do et al., 2013). There are four types of muscarinic ACh receptors (mAChR) which are present in striatopallidal and striatonigral MSNs as well as glutamatergic terminals of cortical projections (Ding et al., 2010; Do et al., 2013).

ACh neurons play a significant role in motor control by modulating striatal output (Perez-Lloret, Peralta & Barrantes, 2016). Some studies have shown that DA release can be triggered by ACh release in nigrostriatal varicosities acting on nAChRs. The opposite effect can be triggered by ACh release activating mAChRs. DA has also been shown inhibit cholinergic interneurons, which become hyperactivated in PD. On the other hand, this cholinergic hyperactivity further potentiates the reduction in dopaminergic activity, making it a vicious cycle (Brichta, Greengard & Flajolet, 2013; Perez-Lloret, Peralta & Barrantes, 2016). The resulting increase in striatal ACh levels is believed to contribute to the development of motor signs seen in PD.

DAT CHARACTERIZATION AND FUNCTION

The dopamine transporter (DAT) is part of a large family of transporters (Na⁺ and Cl⁻ dependent), including other monoamine transporters. The transport process of DAT involves translocating the DA substrate as well as 2Na⁺ and 1Cl⁻ ions across the DA cell membrane (Storch, Ludolph & Schwarz, 2004). It's major physiological role is rapid reuptake of DA from the synaptic cleft, thereby terminating DA neurotransmission. It controls both the intensity and duration of neurotransmission by modulating the dopaminergic concentration in the extracellular space (Storch, Ludolph & Schwarz, 2004). DAT is a bi-directional transporter and is influenced by several factors, including the presynaptic protein alpha-synuclein.

DAT mRNA in the mammalian brain is localized in the cell bodies of DA neurons. In the midbrain, the highest DAT mRNA expression levels are in the SNC and VTA. DAT labelling analyzed in post-mortem human brains indicated that the labelling was most evident in the striatum and moderate in the cell bodies of the SNC, VTA and retrorubral field (RRF). Consistent with this, antibodies highly specific to DAT revealed that the DAT protein was most concentrated in the striatum and nucleus accumbens (Storch, Ludolph & Schwarz, 2004).

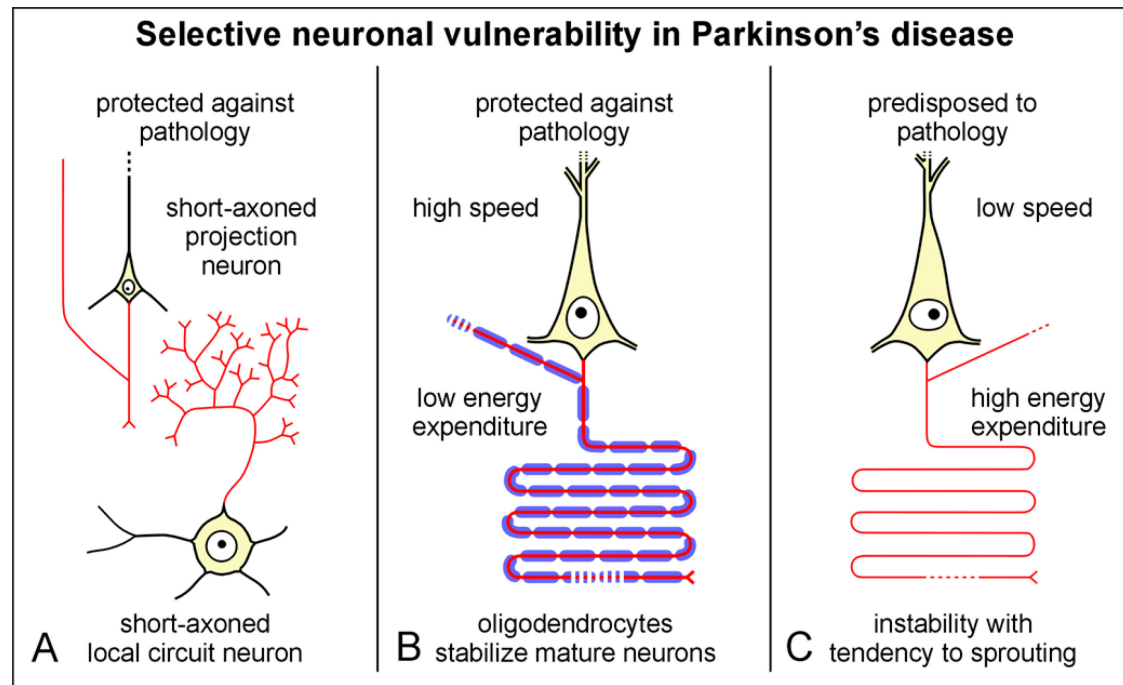
CHARACTERISTICS OF DOPAMINE NEURONS

Cell death in this neurodegenerative disease is associated with neurons that possess long, unmyelinated axons in their region of arborization (Braak et al., 2003a, 2003b; Kapfhammer & Schwab, 1994; Bolam & Pissadaki, 2012), which are primary characteristics of DA neurons. Nerve cells possessing long robust axons that are insulated by thick myelin sheaths however, have been shown to exhibit protection against the formation of Lewy neurites (LN) and Lewy bodies (LB) during the course of PD (Braak et al., 2003a, 2003b; Kapfhammer & Schwab, 1994). Several factors have been suspected to contribute to the potential neuroprotective effects of a thick myelin sheath: the speed of conduction, energy expenditure required for the transmission of impulses, and the interaction of the axon with oligodendroglial cells (*Figure 1*).

A thicker myelin sheath not only increases conduction speed, but also requires less energy for impulse transmission (Braak et al., 2003; Braak et al., 2004). Also, as demonstrated by Kapfhammer & Schwab (1994), the degree of myelination reflects and determines the potential of a brain region for modification of its neural connections. Upon analysis of neurite growth inhibitors (present in myelin) and GAP-43 (marker protein for fiber growth and synaptic plasticity), they demonstrated that myelinated neuronal connections are rather stable, with synaptic plasticity and sprouting actively inhibited by myelination. Poorly myelinated areas however, appeared to be more conducive to sprouting and synaptic plasticity due to the absence of neurite growth inhibitors and increased expression of GAP-43. Their studies also

demonstrated that both the SN and striatal tissue are regions of low myelination (Kapfhammer & Schwab, 1994).

Figure 1: Selective neuronal vulnerability in Parkinson's disease



(Figure 1) Selective neuronal vulnerability in PD. Short-axon projections and local circuit neurons demonstrate increased protection against pathology (A). Projection neurons with long axons and sturdy myelination are also resistant to PD. This is due to the high conductivity, low energy output and superior stability of the parent neuron against axonal sprouting that results from heavy axonal myelination (B). Projection cells possessing long, thin, poorly myelinated axons are among the most vulnerable to PD pathology, due to low conductivity, high energy, and a tendency for sprouting (C). *Figure reproduced with permission of Braak et al., 2004.*

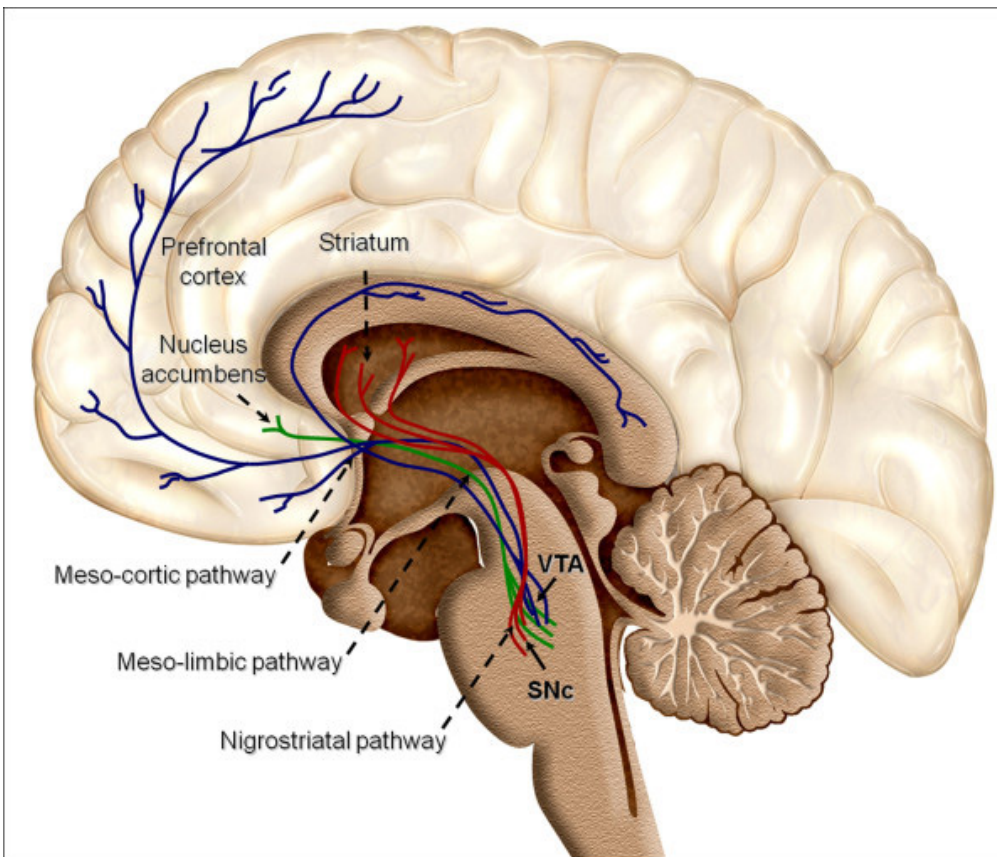
Another reason projection neurons with either no or poor axonal myelination are more prone to pathological sprouting (Kafhammer & Schab, 1994), is that they have an exceptionally high energy turnover. This could result in their being subjected to continuous oxidative stress, which plays a prominent role in the pathogenesis of idiopathic PD (Beal, 1995; Giasson et al., 2000; Scudamore & Ciossek, 2018).

THE MESODIENCEPHALIC DOPAMINERGIC SYSTEM

Dopaminergic neurons, in the adult brain, are localized in the mesencephalon, diencephalon, and olfactory bulb (Arias-Carrion et al., 2010). The majority of these cells however, reside in the ventral part of the mesencephalon. Traditionally, midbrain DA neurons projecting from the SN and VTA were subdivided into four groups: mesocortical, mesolimbic, nigrostriatal, and tuberoinfundibular pathways (*Figure 2*).

The mesocortical pathway consists of dopaminergic neurons from the VTA projecting to the prefrontal, cingulate and perirhinal cortex. The mesolimbic pathway, also referred to as the reward pathway, connects the VTA to the nucleus accumbens and olfactory tubercle of the ventral striatum. This mesocorticolimbic system has been shown to be involved in the modulation of emotion-related behaviour, including motivation, cognitive control and emotional response, and addiction (Smith & Villalba, 2008; Arias-Carrion et al., 2010). The nigrostriatal pathway connects the SNC, via dopaminergic projections, to the caudate nucleus and putamen of the dorsal striatum. This pathway is essential for the initiation and control of movement and is a part of the basal ganglia motor loop. Finally, dopaminergic neurons projecting from the arcuate nucleus in the tuberal region of the hypothalamus to the median eminence make up the tuberoinfundibular pathway. At this site, dopamine release regulates prolactin secretion from the anterior pituitary gland (Smith & Villalba, 2008; Arias-Carrion et al., 2010). From their various nuclei, dopaminergic axons progress medially where they join together and project through the medial forebrain bundle (MFB) to the internal capsule where they then branch off to form synapses in their target regions.

Figure 2: Dopaminergic pathways in the human brain



(Figure 2) Demonstrates the mesocortical (*blue*), mesolimbic (*green*) and nigrostriatal (*red*) pathways. The mesolimbic and mesocortical pathways originate from the VTA and are important for modulating emotion-related behaviors. The mesocortical pathway sends dopaminergic projections to the prefrontal, cingulate and perirhinal cortices. The mesolimbic pathway projects from the VTA primarily to the nucleus accumbens, however it also innervates the amygdala and hippocampus. The nigrostriatal system, originating in the SNc, projects to the caudate putamen and plays an essential role in voluntary movement. *Figure reproduced with permission from Arias-Carrion, et al., (2010).*

These dopaminergic pathways are both anatomically and functionally distinct, though recent evidence is suggesting that this separation is not as exclusive as once thought, and that some of these projections contain cells of origin that are intermixed in the SN-VTA complex (Björklund & Dunnett, 2007). It has been suggested that the caudate putamen may not be the only structure innervated by midbrain DA neurons, and that subsections of other basal ganglia

structures such as the globus pallidus, the ventral pallidum and the subthalamic nucleus also receive innervation from these neurons (Gauthier et al., 1999; Lanciego, Luquin & Obeso; 2012). This gives midbrain DA neurons the ability to also directly modulate the activity of basal ganglia output neurons.

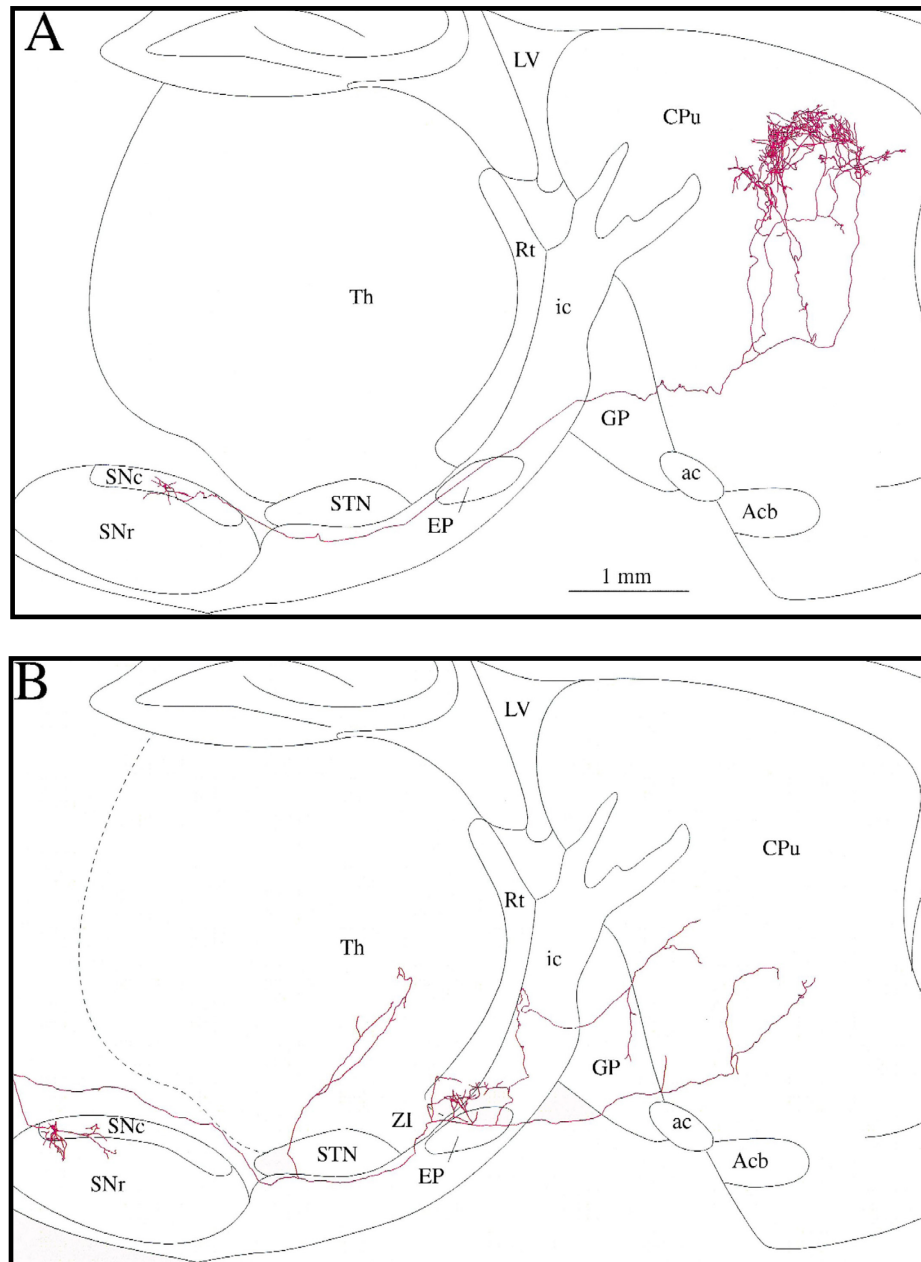
A tracer study of single anterogradely-labeled axons conducted by Gauthier et al., (1999) reconstructed 10 axons whose parent cell body resided in the SNC. They found that these axons appeared to be composed of at least two distinct projection subsystems: those with a profuse arborization in the striatum but poor arborization in the extrastriatal components of the basal ganglia, and those with limited branching in the striatum but a highly patterned set of collateral systems influencing multiple components of the basal ganglia.

Upon characterization of these two axonal subgroups, Gauthier et al. (1999) found that the first type was characterized by only one short thin collateral in the globus pallidus before entering the striatum. It then divided into 4-5 major branches and broke up into many thin and highly varicose collaterals. In contrast, the second subgroup was characterized by axons branching to various extrastriatal structures prior to entering the striatum, where it demonstrated poor arborization. Fibers of this type possessed 2-3 thin and varicose collaterals to either the entopeduncular nucleus, globus pallidus, or both. These collaterals were also shown to arborize in the STN as well. Contrary to the first type, these collaterals displayed infrequent branching and lacked a dense terminal field (*Figure 3*).

This study demonstrated that the nigrostriatal dopamine system is not a monolithic entity as was once thought, but that SNC neurons possess the ability to influence major components of the basal ganglia in a highly specific manner (Gauthier et al., 1999). Other studies have concurred that SNC neurons projecting to the pallidum are largely distinct from those that terminate in the striatum (Smith et al., 1989; Schneider & Dacko, 1991). Not only does extrastriatal innervation appear to play an important role in the functional organization of the basal ganglia, but, when combined with other findings suggesting that fibers terminating in the pallidum display a larger resistance to the MPTP neurotoxin (Smith et al., 1989; Schneider & Dacko, 1991), important functional implications for parkinsonism need to be considered.

Current PD models tend to target the SNC in its entirety, whereas targeting specific subpopulations could be more representative of the disease.

Figure 3: Axonal arborization of single nigrostriatal neurons in rats



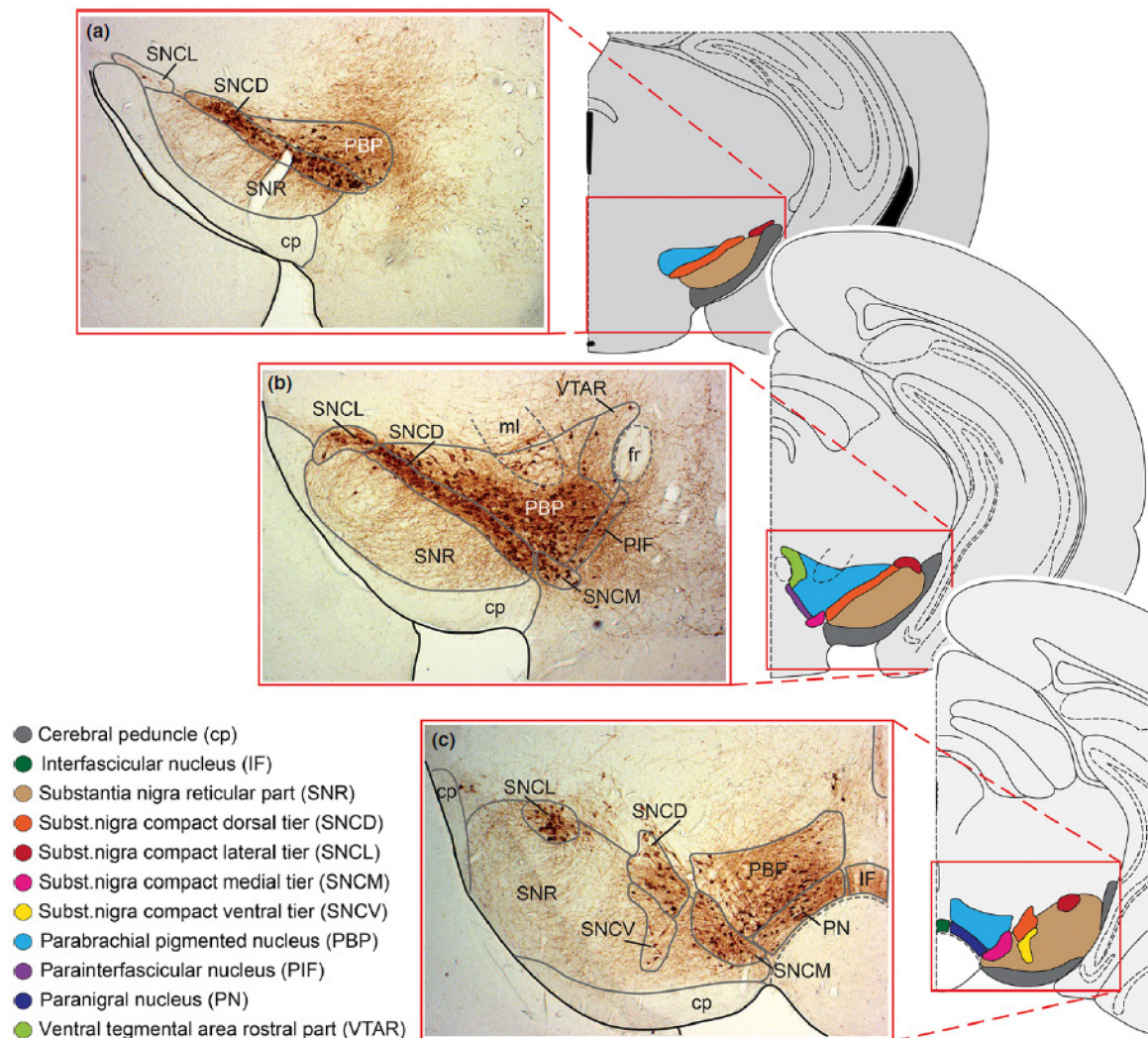
(Figure 3) Schematic representation of the axonal arbor observed in a single rat nigrostriatal DA neuron. The first axonal subgroup type (A) is characterized by a short thin collateral projecting

from the SNC to the GP, then entering the striatum. Once in the striatum, it divided into 4-5 major branches before branching off into many thin, highly varicose collaterals. The second axonal subtype (*B*) sent axonal branches into various extrastriatal regions before entering the striatum where it showed little arborization. This subtype of fibers had 2-3 thin and varicose collaterals that were sent into either the entopeduncular nucleus, globus pallidus and/or the STN. Contrary to the dense arborization observed in the first subtype (*A*), this type had infrequent branching and lacked a dense terminal field. *Figure reproduced with permission from Gauthier et al., 1999.*

VENTRAL MIDBRAIN DOPAMINERGIC NEURONS

Dopaminergic neurons of the ventral midbrain can be subdivided into three categories: A8 (retrosubstantia nigra; RRF), A9 (SNC) & A10 (VTA). The primary composition of these regions is dopamine neurons accompanied by small groups of GABAergic interneurons and glutamatergic neurons (Olson & Nestler, 2007; Smith & Villalba, 2008; Li et al., 2013; Morales & Margolis, 2017). A variety of neuropeptides have been identified in neuronal subsets of the SNC and VTA, however, the main chemical phenotype contributing to the segregation of ventral midbrain regions is the differential expression of calbindin, a calcium binding protein. Exhibiting a strong expression in VTA, RRF and dorsal tier of the SNC (SNC-d), it appears to lack expression in ventral tier SNC (SNC-v) neurons (*Figure 4*). Increasing evidence indicates that VTA and SNC-d DA neurons are much less vulnerable to neurodegeneration than SNC-v neurons, so given the differential CB expression, it has been proposed that its presence could play a neuroprotective role in PD whereas its absence could partially account for the increased vulnerability in SNC-v DA neurons (Smith & Villalba, 2008; Vogt Weisenhorn, Geisert & Wurst, 2016). The varying characteristics of midbrain DA neurons result in differential neurodegeneration of the areas, resulting in complex topographical and regional patterns of cell death. Patterns of nigral and striatal degeneration have recently been closely linked with the levels of expression of CB.

Figure 4: TH-positive neurons in the murine ventral mesencephalic dopaminergic complex



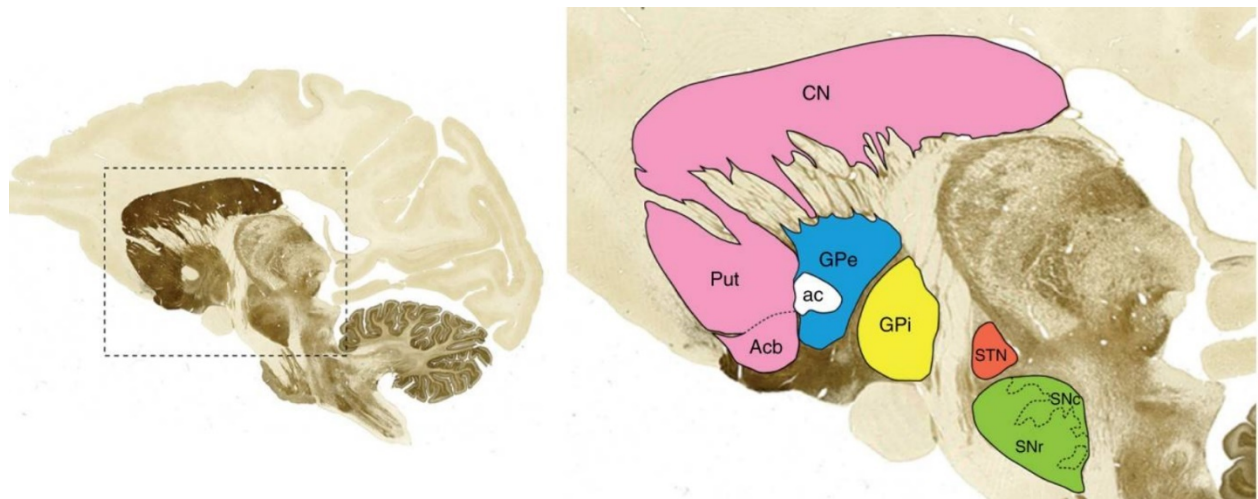
(Figure 4) TH positive neurons are shown in the murine ventral mesencephalic dopaminergic complex, from rostral to intermediate to caudal (A-C). Histological sections are mirror images of the colored sketches the bilateral organization of the complex. *Figure reproduced with permission from Vogt Weisenhorn, Geisert & Wurst, 2016.*

THE BASAL GANGLIA CIRCUIT

The basal ganglia refer to a group of subcortical nuclei responsible primarily for motor control, as well as other roles such as motor learning, executive functions and behaviors, and

emotions. Disruption of this network form the basis for several movement disorders. The basal ganaglia-thalamocortical motor circuit, consisting of the cortex, the basal ganglia and thalamus, is particularly pertinent in PD, as it is clearly perturbed and plays a key role in regulating motor behavior (Lanciego, Luquin & Obeso, 2012).

Figure 5: Schematic Representation of the Basal Ganglia Nuclei



(Figure 5) Schematic representation of the basal ganglia nuclei (*left*) with regions clearly defined (*Right*). Caudate nucleus (CN), Putamen (Put), Accumbens (Acb), external segment of the globus pallidus (GPe), internal segment of the globus pallidus (GPi), Subthalamic nucleus (STN), Substantia nigra pars compacta (SNC), Substantia nigra pars reticulata (SNr). *Figure reproduced with permission from Lanciego, Luquin & Obeso, 2012.*

The basal ganglia includes many regions: the caudate nucleus (CN), Putamen (Put), Accumbens (Acb), external segment of the globus pallidus (GPe), internal segment of the globus pallidus (GPi), STN, SNC and SNr (Lanciego, Luquin & Obeso (2012). The striatum, serving as the primary input of the basal ganglia, plays a significant role in decision-making, particularly in action selection and initiation, which requires the convergence of sensorimotor, cognitive and motivational information (DeLong et al., 1990; Smith et al., 1998). Glutamatergic inputs from the thalamus and cortex are received by the striatum, which in turn, sends GABAergic projection outputs to the GP and SNr. Excitatory synaptic connections are made on MSNs by both cortical

and thalamic inputs. The cortical afferents of these MSNs are from the sensory, motor and associational cortex, whereas the thalamic afferents are from the intralaminar thalamic nuclei (Doig et al., 2010; Do et al., 2013). Various types of neurons form numerous connections in the dSTR, including with cholinergic, dopaminergic and serotonergic axons that strongly innervate the dSTR. Given the complexity of the neuronal circuits in this region, disruption of the signaling in the striatum can cause the movement impairments observed in PD (Lovinger et al., 2010; Do et al., 2013).

THE CORTICOSTRIATAL CIRCUIT: DIRECT/INDIRECT PATHWAY

The basal ganglia have two major pathways regulating motor function: the direct (striatonigral) and indirect (striatopallidal) pathways, both modulated by dopamine. MSNs of the direct pathway exhibit high levels of both D1 and M4 receptor expression, and project to the GPi/SNr. MSNs of the indirect pathway however, exhibit strong D2 and adenosine receptor expression and project to the GPe (Starr, 1998; Do et al., 2013). A balance between these two pathways is necessary to maintain proper functioning and modulation of cortical regions concerned with motor control.

Abnormalities in this circuit are largely responsible for the hypokinetic and hyperkinetic states observed in PD. Basal ganglia-thalamocortical motor circuit alterations can result from the loss of dopaminergic neurons of the SNC. As discussed, the neurodegeneration of the SNC results in depleted striatal dopamine, and this in turn causes a shift in the balance of basal ganglia activity toward the indirect circuit (Starr, 1998; Hill, Wyse & Anderson; 2009; Lanciego, 2013). This shift leads to an increase in activity of the STN, which overstimulates the GPi/SNr. Increased output from these regions results in excessive inhibition of the thalamocortical pathway, which reduces cortical neuronal activation associated with movement initiation (Starr, 1998; Hill, Wyse & Anderson, 2009; Lanciego, 2013). In other words, inhibition of this pathway results in the hypokinetic motor symptoms of PD.

HETEROGENEITY OF MIDBRAIN DOPAMINE NEURONS

Whereas neurons of the SNC and VTA are primarily dopaminergic, increasing evidence indicates that neurons of these regions are more heterogenous than once thought. Dopaminergic neurons from the VTA have long been theorized to play distinct roles in positive and negative reinforcement, decision making, working memory, incentive and stimulus salience, and aversion (Morales & Margolis, 2017). Recent optogenetic experiments have revealed that different functions of the VTA are actually associated with distinct neuronal networks and mediated by a diverse population of VTA DA neurons (Stamatakis et al., 2013; Wang et al., 2015; Yoo et al., 2016). Phenotypic characterization of VTA neurons that subpopulations of TH neurons can vary with regards to biochemical composition, morphological features, functional properties, axonal projections and synaptic connectivity (Li et al., 2013; Wang et al., 2015; Yoo et al., 2016).

Other research has indicated that certain motivated behaviors can be produced by subpopulations of GABA and glutamate neurons in the VTA, independently from dopamine, or even more interestingly, by VTA neurons containing multiple NTs. To date, various subpopulations of VTA neurons have been shown to release dopamine and glutamate from distinct compartments within a single axon (Zhang et al., 2015; Morales & Margolis, 2017). Other subpopulations of these neurons can release glutamate and GABA from a single axon terminal (Root et al., 2014), or co-release DA and GABA from the same vesicle (Berrios et al., 2016). Based on shared characteristics, these various subsets of DA neurons tend to be concentrated in particular subregions of the VTA (Morales & Margolis, 2017).

Typically, dopaminergic neurons can be identified by labeling TH expression, VMAT2 or DAT. Upon closer examination of these heterogenous VTA neurons however, subsets of TH expressing neurons have been discovered that express neither VMAT2 or DAT, which are essential for vesicular repackaging and DA reuptake (Lammel et al., 2008; Stamatakis et al., 2013). In the mouse, subpopulations in the VTA midline nuclei have even been found to express TH mRNA with no detectable levels of TH protein (Yamaguchi et al., 2015; Morales & Margolis, 2017). The purpose of these unique TH neurons in the VTA is currently unknown.

VTA GABA neurons, as identified by GAD or VGAT expression, are distributed throughout the rat VTA and have also been shown to be heterogenous in their composition as well. Various experiments have indicated that only some of these neurons contain corticotropin-releasing factor-binding protein (CRF-BP) or cholecystokinin (CCK) (Wang & Morales, 2008; Morales & Margolis, 2017). Electrophysiological and behavioral studies have indicated that CRF-BP, through its interactions with CRF and CRF peptides, can affect neurotransmission of dopaminergic and GABAergic neurons in the VTA (Wang & Morales, 2008). CCK is a neuropeptide that commonly colocalizes with TH neurons in the VTA but has recently been shown to colocalize with GABA neurons in the VTA lacking TH expression (Olson & Nestler, 2007). Other studies have shown that some subsets of VTA GABA neurons respond specifically to DRD2 or mu-opioid receptor activation, whereas in the past, activation of these two receptors were used to distinguish between dopaminergic and GABAergic neurons, and were assumed mutually exclusive (Margolis et al., 2012).

VTA glutamate neurons have been identified as VTA neurons expressing mRNA encoding VGLUT2. These neurons have been implicated in mediating rapid excitatory signalling and are particularly prevalent within the midline nuclei. This subpopulation of neurons actually outnumbers TH-expressing neurons in some portions of the VTA and have also been found in the hypothalamus (Kawano et al., 2006; Yamaguchi et al., 2011; Morales & Margolis 2017).

Although the majority of VTA neurons signal by releasing DA, GABA or glutamate, recent studies have identified subpopulations of VTA neurons that exhibit combinatorial NT characteristics (Yamaguchi et al., 2011; Li et al., 2013; Zhang et al., 2015; Morales & Margolis, 2017). Whereas much about the mechanisms and function of these combinatorial neurons remain to be elucidated, these neurons have been shown to co-release DA and glutamate, DA and GABA, or both glutamate and GABA.

Preferentially concentrated in the midline nuclei of the VTA, combinatorial TH and VGLUT2 all express the enzyme AADC and are therefore capable of synthesizing DA (Yamaguchi et al., 2011). Electrophysiological and voltammetry studies have confirmed that these neurons do in fact release both glutamate and DA (Li et al., 2013). Some of these neurons

however, have been shown to lack expression of DAT or VMAT2. Upon examination of ultrastructural, biochemical and electrophysiological properties of VGLUT2 inputs from VTA neurons in the nucleus accumbens (nAcc). Zhang et al., (2015) demonstrated a segregation in VGLUT2-TH neurons of dopaminergic and glutamatergic vesicles into different axonal microdomains within a given axon. This study ruled out the theory that nAcc vesicles co-express glutamate and DA and demonstrated that these combinatorial neurons possess two distinct contiguous domains specialized for DA or glutamate release in both mice and rats (Zhang et al., 2015; Morales & Margolis, 2017). Residing in axon terminals, these glutamate vesicles establish asymmetric synapses, commonly associated with excitatory transmission, which lie adjacent to DA axonal segments. This gives individual VGLUT2-TH axons within the nAcc the ability to participate in synaptic or non-synaptic (volume) transmission, meaning. It can therefore provide both excitatory and inhibitory signalling via glutamate and dopamine respectively.

As stated, another form of combinatorial neurons in the VTA have been found to express both TH and GAD. These TH-GAD neurons appear to have a number of subpopulations possessing varied combinations of phenotypes. Some subpopulations have been shown to rarely express VMAT2 and do not appear to exhibit axonal release of DA (Root et al., 2014), others appear to be able to release GABA from the axons of TH-expressing neurons that contain VMAT2 but lack GAD1, GAD2 and VGAT. In cases where VGAT is lacking, it has been suggested that GABA transporters, proposed to be present in DA axon terminal membranes, transport cytoplasmic GABA from the extracellular environment into the cytoplasm of the axon (Tritsch et al., 2014; Morales & Margolis, 2017). In this theory, VMAT2 would then pack cytoplasmic GABA into synaptic vesicles. Unlike the VGLUT2-TH neurons, this does result in the vesicular coexistence of GABA and DA. Also unique to these TH-GAD neurons is the ability of these DA neurons to synthesize GABA directly (Kim et al., 2015; Morales & Margolis, 2017). These neurons therefore, exhibit the ability to release GABA through both synthesis and transporter uptake mechanisms. It is currently not known whether these axons also have the ability to release glutamate.

A subpopulation of non-TH-expressing neurons in the VTA have been identified which co-express VGLUT2, GAD1, GAD2 and VGAT. Some of the axon terminals of these neurons

have been shown to contain both VGLUT2 and VGAT and have the ability to establish both asymmetric (excitatory) and symmetric synapses (inhibitory) (Root et al., 2014). These axons work with postsynaptic neurons possessing both GABA_A receptors and GluR1-containing AMPA receptors to evoke either fast inhibition (followed by excitation) or fast excitation (followed by inhibition), the combination of which could allow pronounced temporal specificity. Finally, a small subpopulation of TH-expressing neurons was identified in the mouse and rat VTA which express VGUT2, GAD1 and GAD2, however it remains unknown to date whether these neurons have the ability to release all three transmitters (Root et al., 2014; Stamatakis et al., 2013; Yamaguchi et al., 2011; Morales & Margolis, 2017).

Interestingly, whereas GABA and glutamate neurons are present in the SNC, there is no evidence to date indicating the presence of combinatorial neurons in this region. Other phenotypic heterogeneity has been identified in SNC neurons however. DAT has been found to be much more highly expressed in SNC neurons as compared to VTA (Blanchard et al., 1994; Storch, Ludolph & Schwarz, 2004), and it is believed that this is one contributing factor to their increased vulnerability. GABA_A receptor subunits have also been found to be heterogeneously distributed at the regional, cellular, and subcellular levels in the human SNC and SNr. The predominant GABA receptor subunits in the SNC are not the same as in the SNr (Waldvogel et al., 2008).

As demonstrated, the characteristics and organization of midbrain DA neurons is much more complex than previously thought. These details however, can allow for much more targeted approaches to pre-clinical PD research and can help elucidate some of the remaining unknowns in the field. Given how little is known about the function or purpose of these heterogenous VTA neurons, one has to wonder if these unique properties contribute at all to the resistance VTA neurons have in PD neurodegeneration.

PARKINSON'S DISEASE

TYPES OF PD

PD is classified into two categories: familial and sporadic/idiopathic. Familial PD occurs in approximately 10% of cases, whereby the onset of disease is due to rare familial genetic mutations (Gillies et al., 2014). On the other hand, sporadic PD is due to exogenous causes influenced by many factors: cellular and molecular processes underlying the degeneration of the nigrostriatal dopamine system, mitochondrial dysfunction, excessive production of reactive oxygen species (ROS), and the formation of pathological protein aggregates (Klein & Schlossmacher, 2007; Gillies et al., 2014). The emerging consensus supported by many researchers in the field today is that PD is a complex, multisystem disease, resulting from the interaction of numerous genetic and environmental factors.

SYMPTOMS OF PD

PREMOTOR PHASE OF PD

The premotor phase of PD is considered as the first stage of symptomology in PD, and occurs when psychopathological processes begin to appear, which can occur many years before the onset of motor manifestations (Cummings & Masterman, 1999; Tolosa, Compta & Gaig, 2007; Zeimssen & Reichmann, 2007; Tadaiesky et al., 2008). Premotor complications include depression, anxiety and cognitive decline can be detrimental to a patient's quality of life. Mood disorders such as depression and anxiety can occur in up to 40% of patients with early stage PD (Tolosa, Compta & Gaig, 2007; Tadaiesky et al., 2008). Whereas these particular mood disorders are relatively well understood on their own, recent studies have started to suggest that the pathophysiology underlying mood disorders in PD may not use the same mechanisms as these disorders in the general population (Lieberman, 2006; Tadaiesky et al., 2008). Although their pathophysiology in the context of PD is not completely understood, dysfunction in striatal, frontal and limbic dopaminergic, cholinergic, serotonergic, noradrenergic and GABAergic pathways are thought to be involved in their genesis (Wolters, 1999; Schrag, 2004).

Dementia and less severe cognitive impairments are also common in PD and can be an important predictor for quality of life. Impaired procedural memory has been associated with striatal alterations, whereas executive dysfunction and working memory impairments have implicated the involvement of the prefrontal cortex-basal ganglia loop (Jackson et al., 1995; Packard & Knowlton, 2002; Lewis et al., 2003; Tadaiesky et al., 2008).

The development and testing of neuroprotective drugs could be greatly facilitated by the ability to diagnose PD prior to motor symptom onset. Ideally, early diagnosis could allow for pre-treatment of the disease to either halt or delay progression. Whereas degeneration of SNC DA neurons is the primary characteristic of PD, neuronal dysfunction or degeneration in other regions within the nervous system likely account for premotor symptoms of the disease (Slow, Postuma & Lang, 2014). One such example is olfactory dysfunction, a key element of the preclinical stage of PD and one of the earliest nonmotor features of the disease. Olfactory dysfunction has been found to occur in an estimated 90% of sporadic PD cases (Doty, Deems & Stellar, 1998; Gagnon et al., 2009; Postuma et al., 2009). To a certain extent, olfactory dysfunction can be predictive of the future development of PD, although given that it is a symptom affected in multiple neurodegenerative diseases there is a lack of specificity. The impairment is rarely complete, is not affected by DA therapies, and has been correlated with abnormal sympathetic cardiac function. Evidence suggests that deficits in cholinergic, noradrenergic and serotonergic function may contribute this olfactory dysfunction (Wolters, 1999; Postuma & Gagnon, 2010).

The specific mechanisms responsible for olfactory dysfunction however, are currently unknown. What is known is that there is significant degeneration and cell loss that occurs in the LC, an area that is responsible for sending noradrenergic projections to the olfactory bulb several olfactory-related structures (Zarow et al., 2003; Doty, 2012). A close relationship has also been established between olfactory function and central cholinergic processes in PD as well as an intimate relationship between serotonin and the olfactory bulb. Marked reductions in both types of these processes have been observed in PD (Scatton et al., 1983; Bohnen et al., 2003; Doty, 2012). Alpha-synuclein pathology is also prevalent throughout the olfactory system in the early

stages of the disease, which is in accordance with the Braak staging system of PD (Braak et al., 2001; Sylveira-Moriyama, et al., 2009).

Another premotor symptom of PD that holds potential for early identification is sleep disorders. In PD, some of these disorders include REM sleep behavior disorder (RBD), insomnia and restless leg syndrome. Manifesting years prior to the appearance of PD motor symptoms, studies have shown that RBD in particular could be used as an important preclinical marker for the disease (Slow, Postuma & Lang, 2014). This is a parasomnia characterized by reduced REM sleep muscle atonia and dream-enacting behaviours. RBD prevalence in PD patients has been estimated to range between 30-50% during the course of the illness. Longitudinal studies have found that RBD patients who develop a neurodegenerative condition will manifest overwhelming symptoms of synucleopathy (Postuma et al., 2009; Slow et al., 2014).

Synucleopathy is found throughout the brainstem and can help explain the increased incidence of olfactory and autonomic dysfunction in RBD patients, as these structures are affected in the early stages of the disease. As such, these two dysfunctions have recently become considered early pre-motor symptoms of PD (Braak et al., 2001; Slow, Postuma & Lang, 2014). Cognition, psychosis and depression also all had increased prevalence in PD patients with RBD. Studies have suggested that the presence of RBD in patients can be used to predict future development of PD, as well as help to identify motor and non-motor manifestations associated with the disease (Postuma et al., 2008, 2012; Slow, Postuma & Lang, 2014).

CARDINAL MOTOR SYMPTOMS OF PD

The four cardinal features of PD include tremor at rest, rigidity, akinesia (or bradykinesia) and postural instability. Freezing and flexed posture are also considered as features of parkinsonism (Jankovic, 2008). Because there is no definitive test for the diagnosis of PD, current diagnostic criteria for PD should include a combination of motor and nonmotor impairments. Studies have estimated that by the time motor impairments become evident in the

progression of this disease, dopaminergic cell death of approximately 80-90% of SNC neurons has already occurred, accompanied by a 50-80% reduction in striatal DA levels (Fearnley & Lees, 1991; Tagliaferro & Burke, 2016).

BRADYKINESIA

Bradykinesia, or slowness of movement, is one of the most common and easily recognizable symptoms of PD. Although its pathophysiology is not completely understood, studies have shown that this is the symptom which best correlates with degree of dopamine deficiency (Lozano et al., 1995; Cutsuridis V & Perantonis S, 2006; Jankovic, 2008). Bradykinesia is a hallmark symptom of basal ganglia disorders and leads to difficulties in the planning, initiation and execution of movement. Recent evidence suggests that the neuropathology responsible for bradykinesia results from a disruption in normal motor cortex activity mediated by reduced dopaminergic function (Lozano et al., 1995; Cutsuridis V & Perantonis S, 2006; Jankovic, 2008). Electromyographic studies of PD patients has demonstrated that the deficit observed in initiation and maintenance of movement has to do with a reduction in muscle force, thought to be caused by deficits in the putamen and globus pallidus (Cutsuridis V & Perantonis S, 2006; Jankovic, 2008).

TREMOR

Another common and easily recognized symptom of PD is resting tremor. Tremors are typically unilateral and are most prominent at the distal part of an extremity. Resting tremors in PD can involve the hands, lips, chin, jaw and legs and tends to disappear during sleep. Studies have indicated that 100% of PD patients had a tremor at some point during the disease; however, the occurrence of resting tremor is highly variable among patients and disease progression (Martin et al., 1973; Rajput, Rozdilsky & Rajput, 1991; Jankovic, 2008). Hughes et al., (1993) reported that resting tremor appeared at onset of disease in 69% of patients, and that tremor was lost in 9% of patients late in the disease.

RIGIDITY

Rigidity is characterized by increased muscle tone which produces increased resistance to movement. This stiffness, caused by an excessive and continuous flexing of the muscles, can cause joint pain and is a frequent clinical manifestation of PD. Rigidity in PD is often accompanied by the “cogwheel” phenomenon, whereby reinforcing manoeuvres increase rigidity during the passive movement of a limb (either proximally or distally), particularly when associated with an underlying tremor (Jankovic, 2008).

CURRENT TREATMENT OPTIONS

Despite tremendous research efforts, the two primary objectives of therapeutic PD research have yet to be achieved: prevention of its onward progression and reparation of systems already damaged (Tagliaferro & Burke, 2016). There is currently no cure for PD and current treatment options only offer symptom management. These treatments include pharmacological therapies, surgical interventions and cell replacement therapies.

PHARMACOLOGICAL THERAPIES

Given that DA cannot cross the blood brain barrier, drug treatment options are aimed at either indirectly replenishing DA stores or mimicking the action of DA. These dopaminergic medications can be effective, for a time, at reducing the cardinal symptoms of PD. Whereas there are certain benefits to all the current PD drug treatments, their limited effectiveness and negative side-effects leave a lot to be desired. One of the most serious downsides to current drug treatments are psychiatric manifestations. Patients with PD are more likely to develop depression than the general population, which can be treated with anti-depressants (Guttman, Kish & Furukawa, 2003). Drug-induced psychosis can be a major management problem however, with patients developing visual hallucinations, paranoia and other psychotic symptoms. These issues can be resolved with the reduction or elimination of certain parkinsonian therapies, which often results unfortunately, in a worsening of the original parkinsonian symptoms. Therefore, given that the only current treatment options available focus

on reducing the burden of symptoms of PD, more research is currently needed in the development of disease-modifying drugs (Guttman, 2003).

LEVODOPA

The most prevalent treatment today is administration of the dopamine precursor levodopa (L-DOPA), which functions to replenish DA stores through uptake into DA neurons and transformation into dopamine via the enzyme DOPA decarboxylase (Blaschko, 1942; Le, Sayana & Jankovic, 2014). L-DOPA is often prescribed in conjunction with Carbidopa, to help prevent it from being destroyed by enzymes in the digestive tract. Whereas L-DOPA is considered the cornerstone for PD treatment, it is most effective at the onset of the disease and approximately 40% of patients with advanced disease develop motor fluctuations and dyskinesias as a result of this medication (Rascol et al., 2000; Lee et al., 2008). Often, within approximately five years of treatment, about half of PD patients will find the beneficial effects of the drug marred by the development of involuntary and debilitating dyskinesias. It is theorized that as the disease develops, compensatory mechanisms become overwhelmed, and the delivery of DA synthesized from L-DOPA becomes dysregulated and pulsatile due to a failing storage capacity (Rascol et al., 2000; Lee et al., 2008). High doses of L-DOPA raise the risk of developing other side effects as well, including confusion, delusions, hallucinations, compulsive behavior, and dyskinesia.

DOPAMINE RECEPTOR AGONISTS

Introduced in the early 1970's as treatments for PD, DA receptor agonists have diverse properties that have the capacity to stimulate DA receptors to produce an antiparkinsonian effect. These agonists fall into two categories: ergot-derived and non-ergot derived. Ergot-derived agonists include bromocriptine, lisuride, pergolide and cabergoline. Non-ergot derived agonists include apomorphine, pramipexole and ropinirole. Each of these agonists have different pharmacological profiles and affinities for DA receptors and subtypes (Stocchi, 1998). Bromocriptine, for example, has been used the longest in treating PD, and is a D2/D3 receptor agonist, as well as a 5-HT₂ antagonist. Apomorphine on the other hand, is a potent and short-acting DA agonist active at D1 and D2 receptors (Goetz & Diederich, 1992; Stocchi, 1998).

Unlike L-DOPA, which requires metabolic conversion to its active form, DA agonists function by acting directly on striatal DA receptors, so their effects therefore, are not dependent on the degenerative state of DA terminals and can partly alleviate the motor symptoms associated with PD (Goetz & Diederich, 1992; Stocchi, 1998). Depending on the DA receptor subtype targeted, a combination of these agonists can be used to alleviate both motor and non-motor symptoms of PD.

COMT & MAO-B INHIBITORS

Another dopaminergic treatment for PD involves a class of drugs which work to inhibit the enzymes responsible for inactivating DA, such as monoamine oxidase-B inhibitors (MAO-B) and catechol-O-methyl transferase (COMT) inhibitors. The action of DA released in the synaptic cleft is primarily terminated by its reuptake and catabolism by the enzymes MAO and COMT. Reduction of DA catabolism by these enzymes has been shown to enhance DA neurotransmission (Napolitano, Cesura & De Prada, 1995). These drugs can be used either as an alternative to or in conjunction with L-DOPA and DA agonist treatments, and they allow DA to last longer in the brain. The use of either of these inhibitors in conjunction with L-DOPA is superior to L-DOPA as a monotherapy at reducing PD symptoms in patients with advanced PD (Talati et al., 2009).

ANTICHOLINERGICS

Anticholinergic drugs, one of the oldest classes of anti-Parkinsonian drugs, can also be used to help reduce tremor and dystonia. However, they have a tendency to induce relatively strong neuropsychiatric and cognitive side effects, so their use should be limited (Ferreira et al., 2013; Brichta, Greengard & Flajolet, 2013). Effective future ACh treatments should focus on stimulation of postsynaptic cholinergic receptors, which could help reduce gait disturbances found in PD.

SURGICAL THERAPIES

There are several functional neurosurgical techniques currently available to help treat the symptoms of PD, including deep-brain stimulation (DBS), ablative surgery and cell

transplantation. DBS is widely indicated as a treatment option when conventional pharmacological measures have failed, and neurological symptoms have become severely disabling (Cenci, Ohlin & Odin; 2011).

DBS is a surgical procedure whereby macroelectrodes, connected to an electrical stimulator, are stereotactically implanted into either the GPi or STN. These are typically the two brain regions targeted for DBS and their stimulation has been shown to be more effective than pharmacological approaches in patients with advanced PD and motor symptoms (Duff & Sime, 1997; Follett, 2004; Cenci, 2011). Amelioration of tremor and rigidity symptoms by this method has been shown to be relatively stable over the long-term, although a gradual decline in hypokinesia has been observed. Neurosurgical risks and side effects include intracerebral bleeding, infections, and misplacement of electrodes. Cognitive worsening is also a risk that is particularly prevalent in older patients. Thalamic stimulation has also proven useful against parkinsonian tremor; however, it has demonstrated little efficacy in treating other PD symptoms (Duff & Sime, 1997; Follett, 2004; Cenci, 2011).

Partial innervation or ablation of some of the other subregions involved in the basal ganglia-thalamocortical motor circuit could also potentially assist in maintaining normal motor function once the nigrostriatal pathway begins to degenerate (Rand et al., 1993; Björklund & Dunnett, 2007). Thalamotomy, pallidotomy and subthalamotomy procedures involve lesioning or ablating the thalamus, lesioning a small portion of the globus pallidus, or destroying the subthalamus, respectively. Given that, unlike DBS, these procedures are irreversible, they are normally not the first surgical option (Rand et al., 1993; Björklund & Dunnett, 2007).

CELL REPLACEMENT THERAPY

Primary cell transplantation is currently the gold standard for cell replacement strategies in the late stages of the disease when pharmacological therapy loses its efficacy (Fricker, Kuiper & Gates, 2012). This method has been extensively studied and has come a long way since early studies demonstrated its potential as a clinical treatment. A number of clinical trials and animal model experiments have provided proof-of-concept for DA neuron replacement as a promising experimental therapy (Fricker, Kuiper & Gates, 2012; Kauhausen, Thompson & Parish, 2013;

Haobam et al., 2015). Results from these experiments and trials have typically been suboptimal with variable functional outcomes.

The predominant focus of cell replacement therapy for PD has been on ectopic transplantation of fetal DA neurons into the striatum as a means to restore neurotransmission. These grafts however, seem unable to restore important aspects of DA circuitry required for basal ganglia output control. DA neurons, for example, lack their normal pattern of afferent input when placed in the striatum which could compromise the reciprocal afferent striato-nigral projections that regulate DA axon arbour size, trajectory and DA delivery (Kauhausen, Thompson & Parish, 2013). The fine-tuning of basal ganglia output and motor function would also be impaired due to the failure of these ectopic grafts to establish dendritic DA control of the SNr.

Homotopic grafts into the site of cell loss however, provide another potential experimental option. Originally this approach was disregarded because it would require extensive axonal growth. It was recently shown however, that DA neurons in homotopic allografts of embryonic ventral mesencephalon can develop long axonal projections along the nigrostriatal pathway and innervate various forebrain targets. It has also been shown that axonal growth and striatal innervation can be significantly increased in rodents with the over-expression of GDNF in the host brain. Recent studies demonstrated that combining GDNF treatment with the use of younger donor tissue can improve motor function, an improvement that is attributed to both local and striatal innervation (Kauhausen, Thompson & Parish, 2013).

While progress is being made in the field, a number of hurdles still remain to be overcome. One important hurdle is the large number of embryonic donors required for a single human graft. In the collection and grafting process, there is also high risk of compromising the viability and functionality of the transplanted cells, causing the patients to suffer dyskinesias following transplantation (Fricker, Kuiper & Gates, 2012). Recovery following cell replacement therapy depends on a number of factors: the source of origin of the transplanted material, the region of brain selected, and the procedure for transplantation. Transplantation of an inadequate

or excessive number of cells and individual differences in disease pathogenesis are also critical factors to consider (Haobam et al., 2015).

Recent advances have been made in an alternative option: grafting of neural stem cells (NSCs). These cells have been defined as multipotent, self-renewing progenitors, possessing an intrinsic capacity to rescue dysfunctional neural pathways (Zuo et al., 2015). Recent studies focusing on host microenvironment has led to significant advances in this therapeutic strategy. The microenvironment of the graft has been deemed critical in determining the fate of donor cells, particularly with regards to astrocytes. Astrocytes, the support cells of the nervous system, participate in blood flow regulation, nutrient transport and modulation of synaptic transmission (Jiao & Chen, 2006; Zuo et al., 2015). In addition, microglia, which are integral in the process of neuroinflammation, have been shown to be detrimental to both host and grafted cells through the release of multiple cytotoxic molecules in the nigrostriatal pathway in PD.

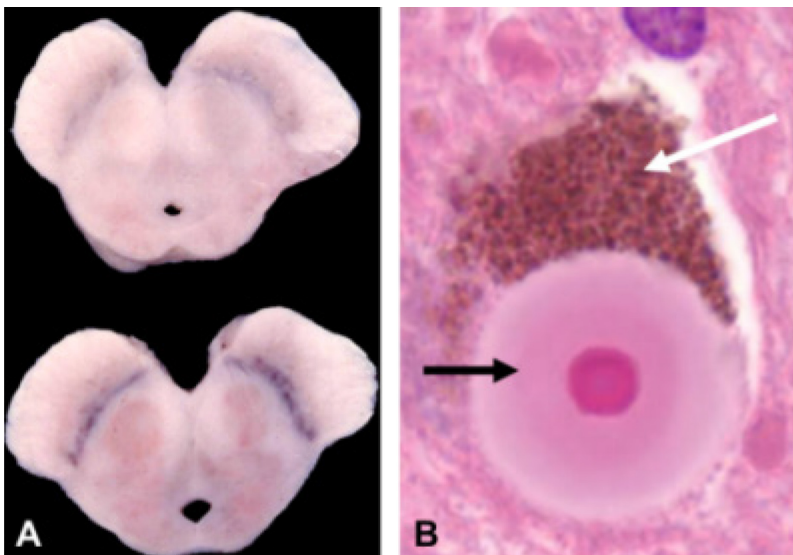
Neuroprotective and behavioral restoration effects have been observed by inhibiting microglia and proinflammatory cytokines. Studies involving human neural stem cells (hNSCs) transplantation therefore, were found to produce a significant neurorescue response, whereby grafted cells not only survived, but proliferated and migrated within the astrocytic scaffold (Harrower et al., 2006; Zuo et al., 2015). Local astrocytes were shown to undergo de-differentiation and acquire the properties of NSCs, resulting in significantly higher expression of host-derived growth factors and inhibition of local microglia and proinflammatory cytokines. These studies therefore, demonstrated that hNSCs transplantation can provide neuroprotection against DA depletion through the recruitment of endogenous cells to establish a favorable microenvironment (Akerud et al., 2001; Zuo et al., 2015). Further studies manipulating and understanding the synergistic interaction between graft and host cells could advance cell-based therapy optimization for PD.

THE PATHOPHYSIOLOGY OF PD

LEWY BODY PATHOLOGY

The neuropathologic process of PD is marked by the presence of alpha-synuclein inclusions in neurons in numerous regions, including the SNC. LBs are morphologically characterized by an accumulation of abnormal filamentous protein inclusions with alpha-synuclein as their major component. They are intracytoplasmic inclusions found within the soma of neurons (*Figure 6*). Other major constituents of LBs include ubiquitin, phosphorylated neurofilaments, PARKIN, molecular chaperones and lipids (Olanow, Stern & Sethi, 2009; Jellinger, 2009; Kalia et al., 2013).

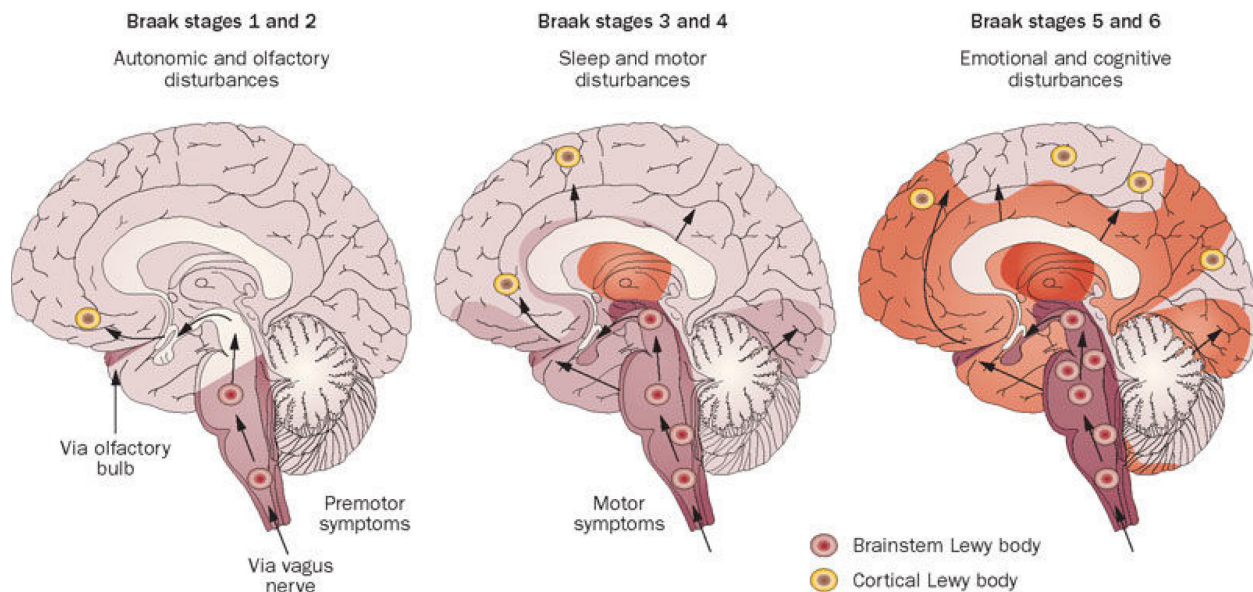
Figure 6: Classic Pathology of PD



(Figure 6) The classic pathology of PD. *Left*: Marked reduction in neuromelanin pigment in the substantia nigra pars compacta (SNC) in a patient with PD (*bottom*) compared with a healthy individual (*top*). *Middle*: Marked reduction in dopaminergic neurons of the SNC of a PD patient (*bottom*) compared with a healthy individual (*top*). *Right*: Surviving DA neuron containing an LB. Note that the LB has a dense core (representing proteinaceous material) surrounded by a pale halo comprised of alpha-synuclein and neurofilaments. *Figure reproduced with permission of Olanow, Stern & Sethi, 2009.*

It has been suggested that neurons with short myelinated axons are more resistant to LB pathology compared to long-range projection neurons with long non-myelinated axons (Kapfhammer & Schwab, 1994; Braak et al., 2003; Bolam & Pissadaki, 2012). As discussed, both these characteristics are present in dopaminergic SNC neurons, making them much more vulnerable to neurodegeneration. In many cases, LB pathology is the first step in this disease continuum followed by a relatively uniform topographical progression of inclusions. This led Braak (2003) and others to generate a topographic synucleopathy map with an accompanying six-stage system of development. The relative uniformity of the progression of this synucleopathy allows for the majority of PD cases to be classified into one of these six stages. In this staging process (*Figure 7*), the earliest signs of alpha-synuclein immune-reactivity seen in the brain is in the dorsal motor nucleus of the vagus nerve and in the anterior olfactory structures (Braak et al., 2003, 2006). Alpha-synuclein inclusions in the SNC first appear in stage 3, and by stage 4, PD-related motor symptoms have been reported, indicating that this is around the time the pre-symptomatic phase gives way to the symptomatic phase of PD. In the final two stages, severe damage can be observed in the autonomic, somatosensory, and limbic systems, and at this point patients will experience the full range of PD symptomology (Braak et al., 2003, 2006). By stage 6 the disease process attains its fullest topographical extent.

Figure 7: Braak Staging System of Parkinson's Disease



(Figure 7) The Braak Staging system of PD. Brainstem LBs are depicted with red dots and cortical LBs with yellow dots. Braak stages 1 & 2 (*left*) involve the appearance of alpha-synuclein pathology beginning in the olfactory bulb and brainstem regions, resulting in premotor symptoms such as autonomic and olfactory disturbances. The onset of sleep and motor disturbances occurs in stages 3 & 4 (*middle*), with a progression of the pathology spreading to the midbrain and basal forebrain. Stages 5 & 6 (*right*) are the most severe stages of PD, with significant increases observed in LB pathology and the development of pronounced cognitive and emotional disturbances. *Figure reproduced with the permission of Doty, 2012.*

Whereas LB pathology is a hallmark of PD, there is increasing evidence suggesting that these inclusions alone do not fully explain the pathogenesis of the disease (Kalia et al., 2013). The first indication of this came about when the presence of incidental inclusions was found during the autopsy of aged individuals without neurodegeneration or clinical signs of PD. Whereas this could be explained away as a pre-symptomatic stage of PD, the presence of LB alone has not been able to be directly linked to neuronal loss.

Whereas these inclusions are related to neurodegeneration in PD, it has been demonstrated that they are also separable from it. For example, when considering autosomal recessive early-onset PD, only a minority of cases have been found to be associated with LBs, pathological or otherwise. Most cases report that this characteristic is lacking. This heterogeneity of neuropathological findings has also been observed in PD resulting from leucine-rich repeat kinase 2 (LRRK2) mutations, one of the most common PD mutations (Ross et al., 2006; Gaig et al, 2007; Corti, Lesage & Brice, 2011). Neurodegeneration in PD patients has been observed both when LB pathology is and isn't present (Forno, 1969; Cookson, Hardy & Lewis, 2008). One interpretation of these observations is that there could be a different form of alpha-synuclein contributing to neuronal toxicity in these forms of PD. Alternately, neuronal dysfunction and loss in PD could occur through mechanisms that are distinct from alpha-synuclein toxicity (Kalia et al., 2013).

ALPHA-SYNUCLEIN FUNCTION/PHYSIOLOGY

Alpha-synuclein is a presynaptic protein and one of its proposed roles is to promote SNARE (soluble NSF attachment protein receptor) complex assembly during synaptic vesicle exocytosis. N-ethylmaleimide-sensitive factor (NSF) is an enzyme involved in membrane

fusion. Whereas its role is not completely understood, it is an important component in the cellular machinery responsible for the transfer of membrane vesicles from one membrane compartment to another. Fusion of the vesicle with the membrane is aided when SNARE proteins on two joining membranes form a tight complex. A number of mutations in the alpha-synuclein gene have been shown to be responsible for early-onset PD, including: point mutations and gene duplications/triplications (Burré, Sharma & Sudhof, 2014).

Concentrated in presynaptic terminals, alpha-synuclein is found in both soluble and membrane-bound states. Its association with synaptic vesicles suggests that its binding to and dissociation from these vesicles may regulate its physiological function. The binding of alpha-synuclein to membranes plays a physiologically significant role. It has been shown in culture to remodel membranes, influence lipid packaging and induce vesicle clustering (Jiang, de Messieres & Lee, 2013; Oubrai et al., 2013; Leftin et al., 2013; Diao et al., 2013; Burré, 2014). Alpha-synuclein assumes different conformations depending on its location: when membrane-bound, it assumes an alpha-helical conformation, whereas in the cytosol it is unfolded and monomeric (Ulmer & Bax, 2005; Burré et al., 2013). It was recently demonstrated that upon membrane binding *in vivo*, alpha-synuclein transforms from a soluble monomeric state to a multimeric state, thereby becoming functionally active. Burré (2014) also discovered that this membrane-bound form of alpha-synuclein chaperones SNARE complex assembly at the synapse, potentially providing protection against neurodegeneration. It therefore makes sense that in its pathological state, mutated alpha-synuclein contributes to the pathogenesis of PD.

ALPHA-SYNUCLEIN PATHOLOGY

One critical question is what leads monomeric alpha-synuclein to form toxic inclusions? Recent findings *in vitro*, using high resolution microscopic techniques, have suggested that the aggregation of two or more monomers leads to the formation of soluble oligomeric species (Walsh et al., 1997; Kalia et al., 2013). Various oligomeric species were observed, however, they all disappeared upon formation of amyloid fibrils. Oligomeric species of alpha-synuclein have the ability to self-associate and form a seed, especially when alpha-synuclein is present at high concentrations (Lashuel et al., 2002; Kalia et al., 2013). Following seed formation,

aggregate growth proceeds rapidly with the further accumulation of monomers or other oligomers. Increased concentrations of alpha-synuclein and subsequent macromolecular crowding are likely the consequence of missense mutations in the SNCA gene (i.e. those associated with familial PD), particularly mutations in which the SNCA gene is duplicated or triplicated. Polymorphisms in SNCA are also risk factors for the development of idiopathic PD (Simon-Sanchez et al., 2009; Fuchs et al., 2008; Kalia et al., 2013).

Some evidence suggests that Lewy body pathology is capable of spreading from neuron to neuron and that alpha-synuclein aggregates may propagate in a prion-like manner. It has been theorized that certain oligomeric species of alpha-synuclein may promote neuronal death by seeding (Danzon et al., 2007; Desplats et al., 2009; Mullin & Schapira, 2013; Kalia et al., 2013). In vivo experiments have supported the theory of prion-like propagation in cases where healthy DA neurons are transplanted into PD brains and they eventually developed Lewy bodies (Li et al., 2008; Hansen et al., 2011).

Mechanisms of alpha-synuclein oligomer toxicity have only recently begun to be elucidated. One of these mechanisms is thought to be impaired proteostasis (protein homeostasis). Cells maintain proteostasis in the cytoplasm through the refolding of misfolded proteins or targeting them for degradation. It has been speculated that cell death occurs when alpha-synuclein oligomers inhibit these critical systems and disrupt proteostasis (Linderson et al., 2004; Kalia et al., 2013). This process is critically important to the maintenance of a healthy cell, and its impairment can lead to endoplasmic reticulum (ER) stress, which has the ability to induce cell death. These findings have been supported with post-mortem examination of human PD brains where chronic ER stress was implicated in neurodegeneration (Smith et al., 2005; Colla et al., 2012; Kalia et al., 2013).

ALPHA-SYNUCLEIN AND MITOCHONDRIAL DYSFUNCTION

Alpha-synuclein and mitochondrial dysfunction have been shown to be very tightly linked in PD pathology. Proper cellular functioning requires an abundant supply of adenosine triphosphate (ATP), which relies upon a pool of healthy and productive mitochondria, therefore, defective mitochondria can be detrimental to cell survival. Nuclear mutations of mitochondrial

proteins and defective mitochondrial DNA (mtDNA) are examples of a couple of factors that can result in defective mitochondria. If left unchecked, these factors can easily result in cell apoptosis (Gorman, 2008; Mullin & Schapira, 2013). Mitochondria have a system for quality control, which involve processes of fission, fusion, and autophagic destruction (mitophagy). Disruption of these processes have been shown to result in mitochondria that display a loss of directed movement, that become swollen and aggregated, and eventually are unable to migrate to their required locations (Deng et al., 2008; Poole et al., 2008; Schapira, 2016). A wide range of neurodegenerative diseases have been linked to impaired fusion/fission dynamics. Mitophagy is an important process whereby damaged or incompetent mitochondria are selectively isolated and removed. Impaired mitochondrial fusion or fission can result in impaired or halted mitophagy altogether (Deng et al., 2008; Poole et al., 2008; Twig et al., 2008).

Numerous studies have shown alpha-synuclein to cause direct mitochondrial toxicity. Studies using various transgenic lines and cells in culture demonstrated that overexpressing alpha-synuclein can result in the appearance of giant mitochondria containing laminated bodies, dysmorphic mitochondria with inclusions and damaged mtDNA, or fragmented mitochondria presumably resulting from reduced mitochondrial membrane fusion (Hsu et al., 2000; Twig et al., 2008; Mullin & Schapira, 2013). Alpha-synuclein has been shown to localize to both the outer and inner membranes of mitochondria with the ability to, upon membrane contact, change conformation from a closed to an open state. Once this incorporation takes place, it leads to fragmented and aggregated mitochondria (Hsu et al., 2000; Twig et al., 2008; Mullin & Schapira, 2013).

ALPHA-SYNUCLEIN AND DOPAMINE

Recently, a number of observations have also been made regarding the impact of increased levels of wild-type or mutant forms of alpha-synuclein on the dopamine system. Studies have shown for example that this mutant protein has the ability to enhance dopaminergic neuronal dysfunction in a number of ways: reducing DA release, modulating TH activity affecting DA metabolism, or causing dopamine-dependent cell toxicity (Xu et al., 2002; Tabrizi et al., 2000; Nemani et al., 2010; Mullin & Schapira, 2013). These effects have been

demonstrated both *in vitro* and *in vivo*. Studies have also shown that intracerebral injection of viruses that overexpress alpha-synuclein can result in strong and significant alpha-synuclein-induced neuropathology and progressive dopaminergic neurodegeneration (Kirk et al., 2002; Decressac et al., 2012; Koprach et al., 2010; Oliveras-Salva et al., 2013). This is in contrast to studies demonstrating that transgenic mouse models of PD do not display significant degeneration of nigral DA neurons nor the DA-dependent motor phenotype (Low & Aebischer, 2012; Oliveras-Salva et al., 2013). A number of studies have reported that rAAV vector-mediated overexpression of either WT or mutant alpha-synuclein, induced 20-80% dopaminergic cell death within 3-8 weeks in rats (Kirk et al., 2002; Decressac et al., 2012; Koprach et al., 2010; Oliveras-Salva et al., 2013).

Overexpression of alpha-synuclein has also been shown to result in decreased quantity of readily releasable vesicles and reduced size of the synaptic vesicle recycling pool. With regards to dopamine specifically, it can reduce dopamine reuptake in dopaminergic terminals and by inhibiting intersynaptic trafficking of vesicles, it causes a reduced DA reserve pool of vesicles (Nemani et al., 2010; Lashuel et al., 2013).

RISK FACTORS OF PARKINSON'S DISEASE

AGE

Age is well-known to be one of the greatest risk factors for PD development (Fearnley & Lees, 1991). This is supported by evidence showing a higher level of LB formation in people over 60, as well as increased expression of alpha-synuclein and decreased expression of TH. Due to these findings and others, it has been hypothesized that parkinsonian degeneration could be an accelerated variant of the normal ageing process (Chu & Kordower, 2007; Collier, Kanaan & Kordower, 2011; Mullin & Schapira, 2013).

Studies have shown that TH expression can be dynamically regulated in response to deafferentation or functional changes. This expression changes during postnatal maturation, and in general, declines with age. Experiments conducted in recent years have demonstrated that there is an inverse relationship between downregulation of the DA phenotype and increased

alpha-synuclein content. This relationship was observed in aged humans and patients with PD (Emborg et al., 1998; McCormack et al., 2004; Björklund & Dunnett, 2007).

ENVIRONMENTAL RISK FACTORS

By far, the most common and agreed upon environmental risk factor for PD is exposure to pesticides and/or herbicides. Despite this, very little is actually known about the degree of pesticide/herbicide exposure required to cause parkinsonism in humans. Rotenone and paraquat, a pesticide and herbicide respectively, are the most commonly studied neurotoxins in relation to PD. As will be discussed in further detail below, these two chemicals have been used in creating animal models of the disease. (Dick et al., 2007)

GENETIC RISK FACTORS

There are multiple genetic factors contributing to familial PD; to date, at least 17 different genes have been identified (Lüking et al., 2000; Bonifati et al., 2003; Valente et al., 2004; Kay et al., 2008). The links or commonalities between familial and sporadic PD however, have only recently become clearer. Many of these genetic mutations causing familial PD are associated with the molecular pathways often affected in the sporadic forms of the disease. For example, a number of the mutated genes responsible for familial PD are also responsible or involved in protecting against mitochondrial dysfunction and oxidative stress (i.e. PINK-1, DJ-1), which are important pathological processes observed in idiopathic PD (Bonifati et al., 2003; Valente et al., 2004).

Among the mutations associated with familial PD, three genes in particular are important to note regarding LB formation: PARK1, PARK2, & PARK5, which encode alpha-synuclein, parkin and ubiquitin carboxy-terminal hydrolase L1 (UCHL1), respectively. Certain missense mutations in PARK1 have been shown to result in amino acid substitutions that can lead to autosomal-dominant PD (Polymeropoulos et al., 1997; Lotharius & Brundin, 2002). Parkin, an E3 ubiquitin ligase, is encoded by PARK2. This enzyme is involved in the degradation of damaged or misfolded proteins by the Ubiquitin-proteasome pathway (UPP). Several mutations in this gene have been linked to autosomal recessive PD with an early age of onset (Leroy et al.,

1998; Hershko & Ciechanover, 1998; Giasson & Lee, 2001). PARK5 encodes the UCHL1 deubiquinating enzyme involved in the UPP and is responsible for cleaving polymeric ubiquitin into monomers. Mutation of this enzyme is believed to result in a partial loss of catalytic activity, which could impact the regeneration of free and reusable ubiquitin following protein degradation (Leroy et al., 1998; Hershko & Ciechanover, 1998; Giasson & Lee, 2001).

The PARK1 mutations associated with PD increase the tendency for the alpha-synuclein protein to form protofibrils and are therefore more likely to form aggregates. PARK2 and PARK5 mutations disrupt alpha-synuclein functions because they are both involved in the UPP (Lotharius & Brundin, 2002). Misfolded or damaged proteins are normally degraded by the UPP. Ubiquitination of unwanted proteins is the initial step in the degradation pathway. This process is initiated by the ubiquitin-activating enzyme E1, which works together with E2 and E3 (encoded by PARK2) enzymes to transfer the activated ubiquitin molecule to the target protein, which is then broken down by a proteasome. UCHL1, encoded by PARK5, recycles the used ubiquitin molecules (Shimura et al., 2000; Lotharius & Brundin, 2002). PD patients with homozygous PARK2 mutations would have a complete loss-of-function of the wild-type protein. These patients have exhibited dopaminergic cell death without the presence of LB, indicating that UPP might play a crucial role in the genesis of LB (Farrer et al., 2001; Lotharius & Brundin, 2002).

TRANSGENIC MODELS OF PD

Over the past 15 years, numerous transgenic mouse models of PD have been developed consisting of mutations in genes identified in familial forms of PD. Some of the more common transgenic models include: synuclein-overexpressing mice, synuclein knockout (KO) mice, Parkin-KO mice, PINK-1 KO and DJ-1 KO mice (Lin et al., 2012; Le, 2014). These models can be useful for examining various hypotheses. However, one major disadvantage is that typically, such mice do not show spontaneous age-dependent loss of DA neurons and large motor dysfunctions, and therefore, do not recapitulate the human disease (Kirk et al., 2002; Decressac

et al., 2012; Koprach et al., 2010; Oliveras-Salva et al., 2013). However, much recent research has shown that a “second hit” is required to reveal a robust phenotype in these genetically-modified mice. This second hit can be, for example, exposure to a neurotoxin such as MPTP or 6-OHDA. These mutant mice typically show higher vulnerability to such toxins (Lee et al., 2017; Ramsey et al., 2010; Sotiriou et al., 2010). Inflammatory conditions, such as induced by the bacterial endotoxin LPS also induces larger loss of DA neurons in mutant mice than in wild-type controls (Bian et al., 2009; Gao et al., 2011; Litteljohn et al., 2018).

PARKIN GENE MODEL

The protein ligase, E3 ubiquitin, is encoded by the Parkin gene. Considered a mitochondrial-associated protein, it has been associated with structures such as the ER, Golgi apparatus, synaptic vesicles and mitochondria (Mullin & Schapira, 2013). Parkin mutations are involved in both familial and sporadic forms of PD. Homozygous alleles of this gene cause early onset familial PD and lead to PD without LB formation. Heterozygous alleles can increase the risk of developing late-onset sporadic PD in which case Parkin is found to localize to LBs (van de Warrenburg, 2001; Schlossmacher et al., 2001; Mullin & Schapira, 2013). Parkin is believed to arrest cell apoptosis through cytochrome C inhibition, although its mechanism is currently unknown. Parkin knock-outs are a popular transgenic model used by researchers today and display significant abnormalities in motor function. Whereas mice with these knock outs do not display degeneration of nigral dopamine neurons, a reduction in their respiratory capacity and elevated increase in susceptibility to oxidative damage do result in DA neuronal damage (Greene et al., 2003; Mullin & Schapira, 2013).

PINK1 GENE MODEL

PTEN-induced putative kinase 1 (PINK1) encodes an insoluble protein which contains a mitochondrial targeting sequence located near the mitochondrial inner membrane. Unlike mutations of the Parkin gene, mutations of PINK1 do result in nigrostriatal cell loss. These mutations cause an autosomal recessive form of PD and can have a number of heterozygous and homozygous pathological variants resulting in a range of Parkinsonian features. Transgenic PINK1 KO mice display fragmented mitochondrial cristae, apoptotic mitochondria and

compromised DA release during burst stimulation (Exner et al., et al., 2006; Kitada et al., 2007; Surmeier et al., 2010). Parkin overexpression has been shown to rescue this phenotype, suggesting a potential common biochemical pathway.

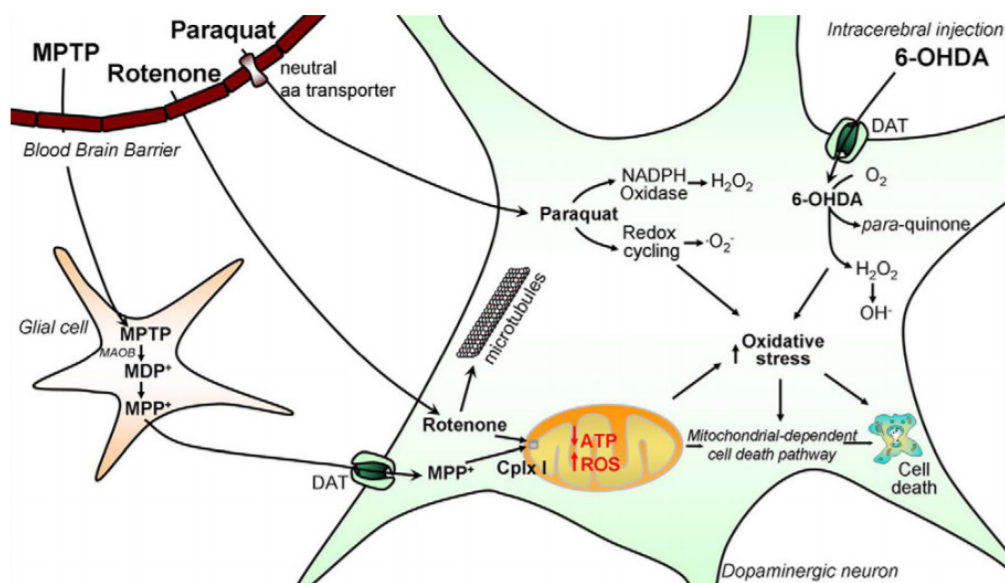
DJ-1 GENE MODEL

DJ-1 is a cytosolic and mitochondrial protein located primarily in the matrix and inner membrane space. In response to oxidative stress however, it has the ability to translocate to the outer membrane of the mitochondria and upregulate the expression of anti-oxidant proteins. Mutations in this gene are a rare cause of autosomal recessive early onset PD. Downregulation of DJ1 has been shown to increase proteasome inhibition and susceptibility to oxidative stress as well as plays a role in alpha-synuclein aggregation inhibition (Schendelman, et al., 2004; Le, Sayana & Jankovic, 2014). Over-expression of DJ-1 has also been reported to protect against MPTP-induced neurodegeneration. Supporting this, DJ-1 KO mice have demonstrated increased sensitivity of cells to oxidative damage, amplified levels of basal oxidant stress in SNC DA neurons and a modest deficit in DA release during burst stimulation (Golderber et al., 2005; Khale, Waak & Gasser, 2009; Surmeier et al., 2010). Whereas locomotor activity is reduced in DJ-1 KO mice, there is no change in DA levels or loss of nigral DA neurons.

NEUROTOXIC ANIMAL MODELS OF PD

There are several neurotoxic animal models of PD currently in use, including: 6-OHDA, MPTP, reserpine, rotenone and paraquat. Unlike 6-OHDA, administered by direct intra-cranial injections, the rest of the neurotoxic drugs are typically administered systemically. Each of these models has particular advantages and disadvantages, and the choice depends on the specific objectives of the experiment.

Figure 8: Mechanism of action of neurotoxins used in PD models



(Figure 8) Mechanism of action of neurotoxins used in animal models of PD. 6-OHDA cannot cross the blood-brain barrier (BBB) and is therefore administered intracranially. It enters DA neurons via the DAT, and following several subsequent reactions, it is oxidized and induces neuronal cell death via the production of reactive oxygen species (ROS). MPTP and rotenone are lipophilic compounds that can cross the BBB and are therefore typically administered systemically. Once in the brain, MPTP is metabolized into MPP⁺ by the MAO-B enzyme in glial cells. This toxic cation then enters DA cells via DAT, where they then accumulate in the mitochondria leading to complex I inhibition. The resulting decrease in ATP levels causes an increase in ROS production and, eventually, activation of a mitochondria-dependent cell death pathway. Rotenone has a similar effect but can also induce depolymerization of microtubules resulting in cell death. Paraquat acts by a different mechanism, whereby it enters the brain via amino acid transporters and catalyzes the formation of ROS. *Figure reproduced with permission of Bové & Perier, 2012.*

THE 6-HYDROXYDOPAMINE (6-OHDA) MODEL OF PD

6-OHDA is a toxin selective for catecholaminergic cells via uptake by DAT. The most widely used neurotoxic PD model in rats since its introduction 50 years ago (Ungerstedt, 1968), 6-OHDA involves stereotaxic injection into the nigrostriatal pathway (Kupsch, 2014). Three mechanisms have been suggested to be involved in 6-OHDA neurotoxicity: monoamine oxidase-induced hydrogen peroxide formation, generation of reactive oxygen species by autooxidation, and direct inhibition of the mitochondrial respiratory chain (Blum et al., 2001; Gonzalez-Hernandez et al., 2004).

6-OHDA is typically either injected into the medial forebrain bundle (MFB), striatum, or more recently, directly into the SN (Hernandez-Baltazar, Zavala-Flores, Villanueva-Olivo; 2017). Whereas the MFB injection, when successful, has been shown to be very effective at inducing cell death and behavioral deficits, the mortality rate in mice tends to be very high with rates as high as 82% having been reported (Lundblad et al., 2004, Iancu et al., 2005; Grealish et al., 2010). Whereas lesion to the MFB has been widely used in rat models, high mortality rates in mice following this procedure has often been a challenge to overcome. This is thought to be due to mice being more prone to high post-lesion complications, resulting in greater mortality rates and weight loss. In the case of Lundblad et al., (2004) however, it was reported that with more vigilant post-operative care and refinement of technique, they reduced the mortality for this procedure to approximately 40%. Injection into the striatum, a much larger target area, tends to yield more reproducible results. However, it has been reported that the number of lesioned animals demonstrating severe neurodegeneration or behavioral deficits can be very low with this method (Grealish et al., 2010). Intranigral injection of 6-OHDA is most often considered the more favorable approach of these three, given that extensive DA neurodegeneration is induced without the high mortality rate associated with MFB lesions (Iancu et al., 2005; Grealish et al., 2010; Hernandez-Baltazar, 2017).

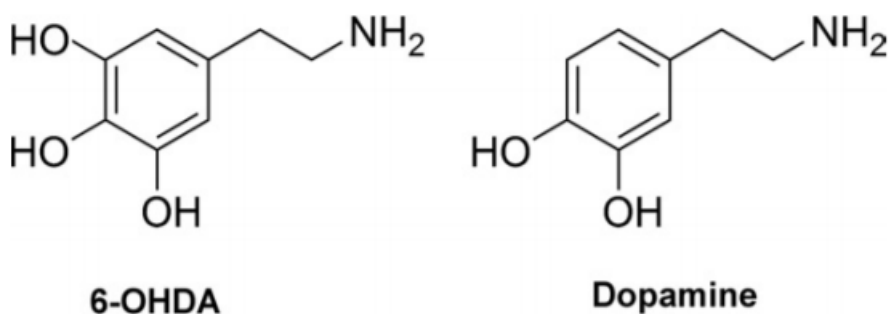
Despite the smaller target area in mice posing increased difficulties with reproducibility, many researchers agree that intranigral injection of 6-OHDA is still the model that holds the most promise for long-term studies in mice (Iancu et al., 2005; Moses et al., 2008; Parish et al., 2008). It has also been argued that the 6-OHDA neurotoxic model is more representative of the human pathology than other PD models because the level of dopaminergic cell loss in the SNC and the pattern of DA depletion in this model are more similar to that observed in advanced human PD pathology (Deumens, Bjorkland & Prickaerts, 2002).

6-OHDA MECHANISM OF ACTION

Intranigral injection of 6-OHDA causes loss of DA cells in the SN via retrograde axonal transport and loss of DA terminals in the striatum via axonal disruption (Hudson et al., 1993). As demonstrated in *Figure 9*, 6-OHDA has a high chemical similarity to catecholamines. As

such, it exhibits a high affinity for the catecholamine transporters, thereby making it a useful drug to cause selective degeneration to catecholaminergic neurons. The deleterious effect of 6-OHDA is largely due to oxidative stress, brought on by the production of reactive oxygen species (ROS) following its uptake by DAT. To increase the targeting specificity of the DA system, 6-OHDA is typically administered in conjunction with a selective noradrenaline reuptake inhibitor, such as desipramine, to block the noradrenaline transporters and inhibit 6-OHDA uptake into their neurons (Deumens, Bjorkland & Prickaerts; 2002).

Figure 9: Comparison of the chemical structures of 6-OHDA and dopamine



(Figure reproduced with permission of Bové & Perier, 2012)

MPTP, RESERPINE, ROTENONE & PARAQUAT

Whereas 6-OHDA is the most commonly used model of PD in rats, MPTP is still the most commonly used PD model in mice (Jonsson, 1983; Bové et al., 2005; Grealish et al., 2010). MPTP, reserpine, rotenone and paraquat are all drugs typically administered systemically. These models have the advantage of negating the need for stereotaxic surgery while inducing neurodegeneration through neurotoxin-induced oxidative and mitochondrial damage (Bové et al., 2005; Grealish, 2010). Given the systemic administration, these models are not as useful however, for functional studies examining lesion-induced behavioral deficits, particularly unilateral parkinsonian deficits. Also, MPTP effectiveness can be strain specific, and can also be transient unless a chronic heavy regimen is used (Sedelis et al., 2000).

Depending on the drug protocol, reserpine can very acutely and near-completely deplete dopamine stores in the brain. However, the strength and speed of this drug can also result in very high mortality rates. Rotenone and paraquat, a common pesticide and herbicide respectively, are used much less commonly than the other aforementioned neurotoxins. When used to induce parkinsonism however, they tend to produce highly variable effects in neurodegeneration levels and mortality rate. These two neurotoxic drugs are therefore much less commonly used as models of PD, and as such, less is known regarding their additional side effects. (Bové et al., 2005; Bové & Perier, 2012).

PARKINSONIAN BEHAVIOR IN UNILATERAL MOUSE MODELS OF PD

Unilateral 6-OHDA injections have long been used to evaluate parkinsonian behavioral symptoms in animal models of PD. Both cognitive and motor deficits can be detected with a wide range of behavioural tests. These tests are well established for severe unilateral lesions (>80% cell loss of SNC), however the results are contradictory and mostly inconclusive for detecting partial unilateral lesions (Carman, Gage & Shults, 1991; Formaguera et al., 1994; Barnéoud et al., 2000; van Oosten & Cools, 2002; Boix, Padel & Paul, 2015).

The most commonly used tests to detect motor impairments after unilateral lesions are the rotarod and cylinder tests. The rotarod allows for measurement of motor coordination, as the subjects are required to keep their balance as long as possible on an elevated rotating rod. The cylinder test allows for two separate measurements, spontaneous rotation and preferential forepaw use. Typical parkinsonian motor deficits include a decrease in motor coordination, spontaneous rotation in the direction of the lesion, and preferential forepaw use of the paw ipsilateral to the lesion. Other relevant behavioural tests include the grip strength test and measures of locomotor activity using actimetry. Reduced grip strength is a common parkinsonian symptom as well as reduced locomotion. Stereotyped behavior is another indicator of a disrupted dopaminergic system. In rodents, this could include repetitive gnawing, sniffing or licking and is dependent on DA receptor activation in the neostriatum (Fuxe, 1979).

The rotational asymmetry observed following unilateral DA depletion is caused by an imbalance in DA activity between the two striata, resulting in the subject rotating away from the hemisphere that has the greater activity (Fuxe, 1979; Fornaguera et al., 1994; Deumens, 2002). Some studies have indicated that this rotational asymmetry can be observed with as little as 50% cell death in the nigrostriatal pathway, though not all studies agree (Carman, Gage & Shults, 1991; Fornaguera et al., 1994; Barnéoud et al., 2000; van Oosten & Cools, 2002; Boix, Padel & Paul, 2015). This asymmetry can be amplified with the use of amphetamines and apomorphine.

Denervation of the striatum induces an upregulation of D2 dopamine receptors causing postsynaptic supersensitivity. Apomorphine, a postsynaptic DA agonist, can be used to induce rotations contralateral to the lesioned hemisphere. This results from activation of D2-supersensitive receptors and preferential activation of DA receptors in the denervated striatum. This apomorphine-induced rotation however, has arguably only been evident in lesions >90%, with the effects of lower levels of cell loss disappearing due to compensatory mechanisms such as compensatory axon sprouting (Deumens, 2002). Studies have reported contradictory results with regards to this however (Carman, Gage & Shults, 1991; Fornaguera et al., 1994; Barnéoud et al., 2000; van Oosten & Cools, 2002). Some studies have also shown that the percentage of dopaminergic cell loss in the SNC correlates with the number of rotations induced by apomorphine, but not amphetamine (Lee et al., 2008). Subcutaneous administration of amphetamine, a dopamine-releasing agent, has been shown to create a DA imbalance favoring the non-lesioned hemisphere, producing ipsilateral rotations.

Other behavioral paradigms that can be used to detect parkinsonian symptoms include: the Morris water maze task for cognitive functions, and the staircase test for fine motor control (Deumens, 2002).

REDUNDANCY OF AXONAL CONNECTIONS IN THE DOPAMINE SYSTEM

There are two major types of intercellular communication in the central nervous system (CNS): point-to-point communication and volume transmission (VT). The former is considered wiring transmission or synaptic transmission and has been the model researchers have used from the beginning. VT, a relatively more novel theory, utilizes communication via the extracellular fluid (ECF) or cerebrospinal fluid (CSF) and involves a large number of cells in the CNS. VT is described as a widespread mode of intercellular communication whereby VT signals move from source to target cells through energy gradients, resulting in diffusion and convection (flow). Axons and terminals involved in wiring transmission allow for rapid and specific transmission of molecules, taking place in a matter of milliseconds. The speed of VT however, is considered much slower, taking from seconds to minutes for the signal to reach its target. These two systems are not considered mutually exclusive and research has indicated that these two systems can integrate by activating various types of receptors located synaptically or extrasynaptically in the plasma membrane.

Recent studies have indicated that the unique anatomical properties of the nigrostriatal dopamine system allow for highly redundant activity of midbrain dopamine neurons. One current theory is that the dopaminergic axonal arbor is so dense and exceptionally large that dopamine release acts not only locally, but also in a diffuse manner affecting neighbouring structures (Vandecasteele et al., 2008; Berretta, Bernardi & Mercuri, 2010; Grace & Bunney, 1983; Arkadir, 2014). It is theorized therefore, that dopamine neurons likely convey their information by VT rather than highly specific point-to-point wiring (Agnati et al., 1968; Descarries & Umbriaco, 1995; Garriss et al., 1994; Zoli et al., 1998). This does not however, lead to a loss of functional specificity as one would expect.

It has been demonstrated that there is redundancy in the information conveyed by midbrain dopamine neurons, which indicates that these neurons form a relatively functionally homogenous population that conveys information through broad wired pathways (Vandecasteele et al., 2008; Berretta, Bernardi & Mercuri, 2010; Grace & Bunney, 1983;

Arkadir, 2014). This may help explain why functionality is preserved following compensatory sprouting.

The mesostriatal DA synapse has been characterized as a synapse favouring NT diffusion into the surrounding extracellular fluid. DA varicosities have been proposed as preferential sites for synthesis, storage and release of DA in VT (Zoli & Agnati, 1996; Zoli & Fuxe, 1998). Supporting the theory of VT, studies have shown that when a releasing stimulus is applied to an intact portion of the striatum following partial DA denervation, there is an increase in DA ECF concentration not only locally, but in the denervated portion of the striatum as well, within a short delay (Schneider, Rothblat & DiStefano, 1994; Zoli & Fuxe, 1998). When this same stimulus is then applied directly to the denervated area of the striatum however, there is no effect. These findings support the theory of diffuse transmission within the dopaminergic system. Studies have also demonstrated increased production and release of DA in the striatum following partial lesion, which has been suggested to result in preferential potentiation of VT (Zoli & Fuxe, 1998).

There are two known types of volume transmission: short distance and long distance. Short distance, or extrasynaptic volume transmission works within a range of micrometers, on local circuits of the CNS, where the monoamine receptors are primarily extrasynaptically located (Fuxe et al., 2016). This type of VT mechanism has been demonstrated in DA, NA, and 5-HT neurons. This helps explain how and why these NT systems have such a powerful role in so many various CNS functions. In contrast, long distance VT occurs within a span of millimeters and involves peptide/protein transmission such as: beta-endorphin, oxytocin, prolactin-like and interleukin-1beta transmission. Borroto-Escuela et al., (2015) proposed the existence of a CNS trophic unit consisting of neurons, glial cells, pericytes and extracellular matrix. They postulated that this neural network survives and functions through integration of VT and wiring transmission signaling in the trophic network by providing the energy necessary for the neuronal network and its brain circuits to operate (Borroto-Escuela et al., 2015; Fuxe et al., 2016). This widespread transmission therefore, has enormous implications for the proper functioning of the CNS.

Aside from DA, VT transmission has also been demonstrated within the glutamatergic and GABAergic systems as well. VT has also been shown to work with various types of cells, for example, neuron-astrocyte units. Synaptic glutamate, working by VT, has been shown to act on extrasynaptic metabotropic glutamate receptors on astroglia resulting in calcium-mediated astrocytic glutamate release into the extracellular space (Del Arco et al., 1996; Fuxe, et al., 2013). In response to synaptic glutamatergic activity, glutamate can then act as an astroglial derived VT signal and contribute to metabolic and trophic adjustments in the neuron-astrocyte unit (Cornell-Bell et al., 1990). Evidence also exists for GABA-mediated extrasynaptic VT acting to modulate astroglia functions and cortical microcircuits (Oláh et al., 2009). Based on the evidence accumulated to date, it is believed that VT may be a large contributor to the dynamic events of synaptic plasticity, and not just for DA.

A central paradigm in neuroscience has been the fact that synapses occur between neurons in the same functional network; they are not randomly distributed. A natural concern, therefore, would be that the anatomical aberrations in the compensatory axonal sprouting process would cause abnormal information flow and therefore not serve as an optimal compensatory mechanism. Previously described studies however, demonstrate that these mechanisms are in fact functional, as demonstrated by normal motor recovery coinciding with striatal reinnervation (Blanchard et al., 1996; Ho et al., 1998; Schneidman et al., 2003; Finkelstein, 2000). The current theory to explain this observation is that the information encoded by midbrain DA neurons is naturally redundant, allowing compensatory sprouting to attenuate the effects of neurodegeneration (Matsuda et al., 2009; Rice & Cragg, 2008; Arkadir, 2014).

It has been demonstrated with both clinical and experimental data that even following a marked degeneration of dopaminergic nigrostriatal neurons (Calne & Zigmond, 1991), DA communication can be maintained in the neostriatum. This can be largely explained by the theory of VT which postulates that under abnormal conditions, like degeneration, DA can diffuse within the striatum to reach supersensitive high affinity DA receptors (Fuxe, 1979). This theory can also help explain why Parkinsonian patients fail to develop motor symptoms in the preclinical stages of PD. Supporting this hypothesis, partial lesion experiments of the

nigrostriatal pathway have demonstrated a compensatory increase in DA release, DA turnover and firing rate in surviving DA neurons (Snyder et al., 1990; Bjelke et al., 1994).

COMPENSATORY MECHANISMS IN THE DOPAMINE SYSTEM IN RESPONSE TO PARTIAL LESIONS

As previously discussed, parkinsonian motor symptoms are not associated with early midbrain DA cell loss because these symptoms only become apparent when the majority of DA axon terminals are lost in the striatum (Stanic et al., 2003; Arkadir, Bergman & Fahn, 2014; Finkelstein et al., 2000). This could be attributed to neuronal reserve but is most likely explained by active compensatory mechanisms that are capable of overcoming the initial cell loss (Arkadir, 2014). To date, adaptations in compensatory axonal sprouting, increased in DA synthesis, metabolism and release have all been reported in studies involving rodent and non-human primates. Upregulation of DA receptors and prolonged clearance time of extracellular DA are also likely to be key factors preventing the motor and cognitive deficits from being observed until late stages of the disease.

COMPENASTORY SPROUTING OF DOPAMINERGIC NEURONS

Recent evidence has suggested that surviving DA neurons of the SNC and VTA have the ability to undergo compensatory axonal sprouting, thereby reinnervating the partially denervated striatum (Finkelstein, 2000; Lee et al., 2008; Arkadir, 2014). Whereas little is known about the mechanism of action involved, studies have shown sprouting to occur in the caudate putamen (CPu) of the rat following a 6-ODHA lesion to the SNC. Finkelstein et al., (2000) showed increased terminal arborization manifested by a substantial increase in collateral branching and axonal varicosities, both of which correlated with the degree of cell loss following partial lesion to the SNC. They demonstrated that the density of DAT in the dorsal tier of the CPu was unchanged from control values until SNC cell loss was >70% (Finkelstein, 2000). They also found that the morphology of newly formed varicosities was altered, being larger and containing more vesicles as compared to control rats. These morphological changes likely explain the late onset of symptoms observed in PD.

Whereas axonal sprouting quantitatively compensates for the loss of DA varicosities, the original organization of the nigrostriatal network is not preserved. It has been suggested that after sprouting, a single axon can extend over the whole rodent dSTR and thus affect a much larger subpopulation of striatal neurons (Blanchard et al., 1996). This theory is supported by the fact that compensatory sprouting in the dSTR following 6-OHDA lesion to the SNC has been observed in VTA DA neurons, whereas normally these neurons would only project to the ventral striatum (Ho et al., 1998; Schneidman et al., 2003; Arkadir, 2014).

A temporal association has been observed between axonal sprouting and motor recovery. Stanic et al. (2003), demonstrated spontaneous behavioral recovery following reconstitution of TH activity in the striatum, suggesting that the newly sprouted axons are physiologically functional. Following an initial decrease in the density of TH positive varicosities in the dSTR in partially lesioned rats, the density of TH positive varicosities started to increase within 8 weeks, peaking at 16 weeks. Motor recovery was observed along the same time course (Stanic et al., 2003). Rats with induced lesions of >75% however, did not demonstrate motor recovery at any time point. These results have been replicated in mouse models as well (Kim et al., 2011). It has also been reported that the degree of dopaminergic axonal sprouting resulting from partial lesions is linearly proportional to the degree of cell death in the SNC (Finkelstein et al., 2000; Stanic et al., 2003; Kim et al., 2011).

It is suspected that once neuronal cell loss in the SNC has surpassed a certain threshold, compensatory mechanisms start to fail; once the axonal arbor of a given DA neuron has surpassed a certain point, it may no longer be able to fully reinnervate the striatum and keep up with the metabolic demands. It is at this point significant deficits in cognitive and motor function become apparent. In the study conducted by Finkelstein et al., (2000), when the lesion size exceeded 75%, surviving SNC neurons were incapable of increasing their axonal arbor to compensate for the loss. It is also likely that once the metabolic needs have surpassed a certain threshold, these compensating neurons undergo accelerated cell death due to elevated ROS stress. This compensatory sprouting then is likely a double-edged sword: it is an efficient compensatory mechanism early on in the disease, however, it may lead to accelerated cell death in later stages of the pathology (Bolam & Pissadaki, 2012; Arkadir, 2014).

INCREASED DOPAMINE RELEASE AND METABOLITE FORMATION

Another proposed compensatory mechanism is increased DA release from residual DA neurons. This phenomenon has been reported in rodents, non-human primates and parkinsonian patients (Bernheimer et al., 1973; Altar et al., 1987; Hefti, Melamed & Wurtman, 1980; Zigmond et al., 1984). This reported increase appears to result from enhanced synthesis and release of DA, as well as a decrease in its inactivation. It has been suggested that increased DA turnover could be, in part, caused by a reduction of the inhibitory influence of presynaptic autoreceptors. This may potentially permit the nigrostriatal bundle to continue regulating striatal function, despite extensive damage, by increasing the area of influence of the residual DA neurons (Synder et al., 1990).

Other studies have shown that surviving DA neurons react to partial lesioning of the nigrostriatal system by increasing metabolite formation. The first increases were found to occur in the striatum at approximately 40-60% dopaminergic cell death of nigrostriatal neurons, however, severe lesions resulted in increases of up to 250% metabolite formation per neuron in the lesioned hemisphere (Hefti, Melamed & Wurtman, 1980; Zigmond et al., 1984). This increased metabolite production reflects increased DA synthesis, turnover and release (Hefti, Melamed & Wurtman, 1980).

As demonstrated, the relation between experimental lesion size and neurological deficit cannot fully be accounted for by simple redundancy in the nigrostriatal system (Creese & Snyder, 1979; Calne & Zigmond, 1991). Zigmond et al., (1984) and others have demonstrated that the increased release of DA was accompanied by a greatly reduced capacity for its inactivation by high-affinity uptake. Loss of reuptake sites due to the loss of dopaminergic terminals in the striatum, combined with the increased release of the NT, appears to result in a restoration of the normal influence of dopaminergic cells in the SN over their striatal targets (Zigmond et al., 1984). Also, given that these adaptations did not occur until the cell death of approximately half of nigrostriatal neurons demonstrates that these compensatory mechanisms are sufficient to maintain dopaminergic neurotransmission following moderate degrees of terminal loss.

INCREASED DOPAMINE RECEPTOR SENSITIVITY

Another compensatory process observed in the striatum following nigrostriatal damage is an increase in the sensitivity of striatal cells to DA. Supporting this, studies which interrupt the nigrostriatal bundle have shown a proliferation of striatal DA receptors (Snyder et al., 1990). This adaptation however, does not appear to occur until approximately 75-90% of the striatum is denervated, at which point these changes occur over the span of several weeks (Zigmond & Sticker, 1980; Creese & Snyder; 1979; Zigmond et al., 1984). This could suggest that a large number of DA terminals in a given projection field may only have minor consequences on DA delivery to postsynaptic receptors. This also suggests that there are likely differences in the compensatory mechanisms initiated depending on the stage of disease progression.

In addition, a study conducted by Bjelke et al., (1994), provided electrophysiological evidence of DA-like action in denervated dopaminoceptive cells following near-complete unilateral lesions to the SN and systemic administration of D-amphetamine. They obtained this result despite the fact that no increased levels of extracellular DA were found in the denervated striatum. This effect could be explained by the DA receptors becoming supersensitive on the lesioned side and are therefore reacting to very low concentrations of DA.

Based on their results, they also postulated that the majority of DA found in the lesioned neostriatum originated from the unlesioned hemisphere and, via diffusion, reached the CSF and the denervated neostriatum on the contralateral side. This theory is supported by the fact that a very marked disappearance of DA terminals in both the neostriatum and ventral striatum on the lesioned side was observed in these subjects. They also demonstrated that DA given in the lateral ventricle can diffuse extraneuronally into the striata bilaterally (Bjelke et al., 1994).

Taken together, these studies provide strong evidence for the restoration of DA communications in a strongly denervated striatum occurring by VT via long distance diffusion in the CSF. It also follows then that DA-releasing agents may, in part, exert their effect in Parkinsonian patients by releasing DA from intact DA neurons and diffusing through the CSF to act on supersensitive neostriatal DA receptors.

Whereas much has been learned to date about these various compensatory mechanisms in rodents and non-human primates, information is lacking with regards to the human pathology of PD. The largest limitations in human studies of PD, particularly the early stages, has been post-mortem analysis. In most cases, by the time PD patients can be autopsied to analyze neurobiological changes resulting from the pathology, the disease is often in its last stages. The evidence provided for human compensatory mechanisms have therefore been limited. More recent studies utilizing various brain imaging techniques however, have brought new data to light. Human studies have now found evidence of various compensatory mechanisms taking place in earlier stages of the disease, such as: compensatory hyperactivation in the inferior parietal cortex and superior frontal gyrus, downregulation of DAT and upregulation of decarboxylase activity as well as compensation of TH mRNA levels (Joyce et al., 1997; Adams et al., 2005; Gerrits et al., 2015). Also, dopaminergic sprouting has been observed in human PD patients following implantation of autologous adrenal medulla or fetal mesencephalon into the striatum (Hornykiewicz & Kish, 1987; Bjorklund & Lindvall, 2000; Hirsch, 2000; Porritt et al., 2005).

There is reason to believe that these compensatory mechanisms come at a cost, however. Enhanced DA turnover could result in the accumulation of toxic by-products of DA oxidation, thereby contributing to the ultimate degeneration of surviving elements of the nigrostriatal bundle. Impaired removal of DA from the synaptic cleft, due to the loss of terminals possessing active reuptake mechanisms, could also potentially result in the accumulation of toxic substances in the extracellular space. Also, as stated, as the axonal arborization size of these compensating DA neurons increases, so does their bioenergetic demands and levels of oxidative stress. At a certain point, the vulnerability of these neurons is increased to the point where these secondary stressors cause them to undergo rapid degeneration. The vicious circle created by all these events would therefore, in the long term, generate more injury to both dopaminergic and nondopaminergic cellular components (Calne & Zigmond, 1991).

NON-DOPAMINERGIC COMPENSATORY MECHANISMS

The dopaminergic system is not the only system capable of undergoing compensation. A lot of studies have demonstrated strong ties between DA depletion and the serotonergic, GABAergic and glutamatergic systems (Berger & Glowinski, 1978; Arai et al., 1994; Bezard, Bioulac & Gross, 1998; Schroeder & Schneider, 2002; Maeda et al., 2003). The serotonergic system appears to play a particularly significant role in compensating for DA loss, especially in cases of severe DA depletion. This becomes especially relevant in cases of neonatal rats receiving 6-OHDA or MPTP injections, which will be discussed in the next section.

COMPENSATORY MECHANISMS OF THE SEROTONERGIC SYSTEM ARE CLOSELY TIED TO DOPAMINERGIC DENERVATION

The serotonergic system appears to have the ability to compensate for DA depletion through a number of mechanisms, including: sprouting, hyperinnervation, DA production, and DA uptake (Berger & Glowinski, 1978; Luthman et al., 1987; Arai et al., 1994; Gagnon et al., 2016). It has been suggested that serotonergic sprouting of terminals may occur in response to local dopaminergic denervation (Stachowiak et al., 1984; Luthman et al., 1987). A detailed stereological investigation in MPTP-treated monkeys conducted by Gagnon et al., (2015) revealed the highly plastic nature of the 5-HT striatal afferent projections, particularly in the absence of striatal DA. They demonstrated the proliferative and adaptive changes that take place in ascending axonal projections. Significant increases in the quantity of 5-HT axon varicosities were found in the striatum of these monkeys, particularly in the sensorimotor area of the striatum (2-fold increase) where DA denervation was most severe.

Interestingly, research has suggested that 5-HT neurons innervating the striatum may improve L-DOPA action through the conversion of exogenous dopa to DA and its subsequent release from 5-HT neurons as a false NT (Aria et al., 1994; Maeda et al., 2005; Kish et al., 2008). Animal studies have indicated that, following dopaminergic lesions, DA can be stored and released by 5-HT neurons (Aria et al., 1994; Maeda et al., 2005). Berger & Glowinski (1978) demonstrated that 5-HT nerve terminals have the ability to uptake DA from the extracellular

space and help normalize extracellular DA concentrations. On a more functional level, rat studies have shown that a 20-50% loss of striatal 5-HT is associated with increased spontaneous locomotor activity (Carter & Pycock, 1979; Schwarting & Carey, 1985; Kish et al., 2008). In human PD patients, strong evidence of serotonergic disturbance was provided by the observation of significantly reduced striatal levels of all key serotonergic markers (Kish et al., 2008).

GABA & GLUTAMATERGIC COMPENSATORY MECHANISMS

Compensatory effects have also been observed in GABAergic and glutamatergic systems in response to DA depletion. A number of studies have suggested that these systems, including the serotonergic system, have the ability to completely compensate for DA depletion when DA levels are depleted 95% or more (Schneider & Rothblat, 1991). Regarding the GABAergic system, measures of pallidal activity including basal GABA levels, pallidal neuronal responsiveness to sensory stimulation and pallidal GAD mRNA expression have all found to be altered in symptomatic MPTP-treated cats. Once these animals are functionally recovered from MPTP-induced parkinsonism however, all measures of these activities return to normal (Rothblat & Schneider, 1995; Schroeder & Schneider, 2001, 2002).

Also, glutamatergic compensatory mechanisms have been shown to be active at the end of the pre-symptomatic period and beginning of the symptomatic period of PD (Bezard, Bioulac & Gross, 1998). This has been theorized to be partly due to SNC excitatory afferents activating remaining DA neurons. Excitatory glutamatergic inputs are received by the SNC from the cortex and STN. Both glutamatergic and cholinergic excitatory inputs from the pedunculopontine tegmental nucleus (PPN) are also received by the SNC. These inputs have been proposed to be involved in compensatory effects, given that the STN-SNC pathway has been shown to be hyperactive in PD (Bergman et al., 1994; Miller & Long, 1987). A study by Bezard, Bioulac & Gross (1998), reported behavioural modifications induced by the injection of a glutamatergic antagonist into the SNC of monkeys with little to severe damage to the SNC. Injection into the first group of asymptomatic monkeys induced parkinsonian motor abnormalities and increased the severity of clinical signs in already symptomatic monkeys. Their results suggest that,

glutamatergic inputs to the SNC are likely implicated in compensatory phenomena at various stages of experimental parkinsonism (Bezard, Bioulac & Gross, 1998).

We have demonstrated that dopaminergic compensatory mechanisms have the ability to compensate up until DA stores are depleted by 80% or more. It is possible therefore, that at the extreme end of DA loss, these other systems are able to undergo alterations that allow them to help compensate for the loss. There must however, be other factors at play, because whereas recovery of parkinsonian symptoms has been demonstrated through compensation by these other systems, in general, many rodent models of PD do still exhibit significant cognitive and motor disturbances following severe DA depletion.

COMPENSATORY VARIATIONS BETWEEN NEONATAL AND ADULT LESIONS OF THE NIGROSTRIATAL PATHWAY

The timepoint in the developmental process at which DA-depletion occurs has been shown to be an important factor to consider when evaluating neurochemical and behavioural adaptations. Early studies have demonstrated a number of neurobiological differences in mice lesioned as neonates as opposed to adults. These alterations have been evident in DA-receptor supersensitivity and subsensitivity, in locomotor hyperactivity, and compensatory changes in the serotonergic system. To our knowledge, this information has not currently been published for mice.

ADAPTATIONS OF THE DOPAMINERGIC SYSTEM IN LESIONED NEONATAL MICE

Dopaminergic lesions in neonatal rats has been shown to differentially regulate D-1 and D-2-like receptors, as compared rats lesioned in adulthood. For instance, Neal & Joyce (1992) demonstrated that lesions of the DA system in early postnatal development have different behavioural consequences to lesions made in adulthood. They found that lesions created in rats at this early age resulted in both D1 and D2-receptor agonist supersensitivity and an accompanying subsensitivity to both D1- and D2 antagonists. A loss of D1 receptors (10-15%)

was also observed in the striatum of these rats. This decrease in D1 receptors would appear to contradict the idea of increased sensitivity to D1 agonists, however this alteration in receptor density is believed to reflect the developmental organization of the rat striatum (Neal & Joyce, 1992).

Another differential aspect between neonatal and adult 6-OHDA lesions pertains to the pattern of D1 receptor loss (Mailman et al., 1983; Neal & Joyce, 1992). Neonatal lesions appear to induce D1 receptor loss within the patch compartment of the striatum, whereas adult lesions affect the receptors located in the matrix compartment. Also, in adult 6-OHDA lesions, D2 receptor binding is increased in the lateral striatum, which has been suggested to underlie the supersensitivity to D2 agonists and behavioural recovery these rats typically display.

With regards to the behavioural ramifications of these alterations, some early studies demonstrated that intraventricular injection of 6-OHDA into neonatal rats resulted in hyperactivity of these subjects when they were adults (Miller et al., 1981; Erinoff & Snodgrass, 1985). In the study conducted by Miller et al., (1981), neonatal pups were injected with 6-OHDA, and locomotor activity was monitored from P14-32 and P56-62. These results were compared with subjects that received the same 6-OHDA injections as adults. Various doses of 6-OHDA were tested on both neonatal and adult rats, and it was demonstrated that the extent of DA depletion was proportional to the level of locomotor hyperactivity. This hyperactivity was only observed in rats receiving this treatment as neonates, not as adults. Between 2-3 weeks of life, 3-5-fold increases in locomotor activity were observed relative to controls. From here, locomotor activity decreased to approximately 2-3 times the control levels between P24-32. In neonates receiving the lowest doses of 6-OHDA, this hyperactivity was demonstrated to be transient (return to normal by P56), whereas this increase became permanent in subjects receiving the highest doses (remaining at 3-4 times control values at P62). These results demonstrate that locomotor hyperactivity resulting from 6-OHDA is dependent on both the stage of development at the time of DA neuron destruction as well as level of DA depletion. These results also support current theories of compensation, in that DA neurons spared by 6-OHDA contribute to the restoration of normal locomotion in lesioned rats.

ADAPTATIONS OF THE SEROTONERGIC SYSTEM IN LESIONED NEONATAL MICE

Studies focusing on the involvement of the serotonergic system in the pathology of PD have demonstrated that this system is much more intricately tied to the dopaminergic system than once thought. Studies examining the effects of neonatal DA depletion on the serotonergic system have shown that, like DA, not all compensatory processes are the same for neonates as for DA depleted adult rats. Hyperinnervation of the dSTR, elevated 5-HT levels, compensatory sprouting, changes in 5-HT receptor-mediated signaling and the assumption of dopaminergic roles are just a few of the adaptations that have shown differential compensatory effects depending on the time point of development that DA transmission in the striatum is interrupted (Breese et al., 1984; Stachowiak et al., 1984; Berger et al., 1985; Snyder et al., 1986; Luthman et al., 1987).

Interruption or loss of striatal DA transmission in early rodent postnatal development has been reported to result in a drastic compensatory increase in 5-HT innervation to the dSTR (Breese et al., 1984; Stachowiak et al., 1984; Bishop et al., 2004). This hyperinnervation effect is the result of neonatal DA depletion inducing raphestriatal axons to increase 5-HT innervation to the caudal striatum, as well as collaterals sprouting from these neurons into the more sparsely innervated rostral striatum (Berger et al., 1985; Snyder et al., 1986; Luthman et al., 1987; Bishop et al., 2004).

A study by Breese et al., (1984) demonstrated that striatal levels of serotonin were elevated in mice treated neonatally with 6-OHDA. Furthermore, some studies have reported that serotonin-containing neurons in neonatal 6-OHDA-treated rats are capable of undergoing sprouting, whereas adult 6-OHDA-treated rats were not (Stachowick et al., 1984; Breese et al., 1984). DA-depleting brain lesions in 3-day old neonates has been shown to result in a nearly 4-fold increase in serotonin content of the rostral striatum 1-4 months following the lesion (Mailman et al., 1983; Berger et al., 1985). This elevation in striatal 5-HT was associated with increased 5-HT uptake and increased proliferation of these terminals in the adult striatum of neonatally DA depleted mice. Some of these compensatory alterations have recently been observed in DA depleted adult rats, which is contradictory to the earlier literature. The reason

for this discrepancy has not yet been elucidated (Mailman et al., 1983; Stachowiak et al., 1984; Zhou, Bledsoe & Murphy, 1991; Guerra, Liste & Labandeira-Garcia, 1997).

As with DA receptor expression, variations in 5-HT_{2A} receptor expression has also been observed between neonatal and adult lesions. Results obtained from various studies has led researchers to suggest that there is a functional significance to 5-HT_{2A} receptor plasticity in the dSTR which may influence motor control mechanisms of the DA-depleted rodent. 5-HT₂ receptor-mediated signaling is believed to be another contributing factor to spontaneous hyperactivity in neonatally DA-depleted rats (Luthman et al., 1991). In rats exposed to DA depletion as adults, this depletion has also been shown to foster D1 receptor-mediated hyperlocomotive behaviours (Bishop et al., 2003, 2004). Due to the fact that DA and 5-HT containing neurons appear to have similar effects on striatal interneurons, it has been proposed that 5-HT terminals may assume dopaminergic-mediated functions, thereby contributing to partial behavioural recovery observed in neonatally-lesioned rats (Stachowiak et al., 1984; Maeda et al., 2003).

VULNERABILITY OF SNC DOPAMINE NEURONS

One of the most critical questions in the PD field as of late has been why DA neurons of the SNC show such a select vulnerability in PD. A definite answer to this question has yet to come, but multiple hypotheses have been considered. As stated, unlike in humans, PD does not spontaneously develop rodents, however, a large contributing factor to this is likely the difference in number, size, and complexity of DA neurons across species. In addition, the unique morphological characteristics of SNC DA neurons, elevated bioenergetic demands and oxidative stress have all been shown to contribute to their increased vulnerability.

NUMBER, SIZE AND COMPLEXITY OF DA NEURONS ACROSS SPECIES

The size and complexity of the dopaminergic system in primates is larger compared to rodents. In mice, the number of TH-positive neurons in the SN of each hemisphere is approximately 12,000, as compared to ~20,000 in the VTA (Moss & Bolam, 2009; Brichta &

Greengard, 2014). In contrast, humans have between 400,000-600,000 DA neurons, with more than 70% of these neurons located in the SNC. This difference is not only remarkable with regards to the number of neurons, but also with their level of complexity and innervation. When DA neurons were individually analyzed in the rat brain, it was discovered that the average total length of the axon in the striatum is around 47cm, and its arborization can extend to occupy up to 5.7% of the volume of the striatum (Matsuda et al., 2009; Moss & Bolam, 2009). When considering the number of synapses that are formed in the striatum, this becomes even more impressive. It has been estimated that a single DA neuron can give rise to between 170,000 and 408,000 synapses in the striatum. To put this in perspective, DA neurons of the VTA are estimated to give rise to between 12,000-30,000 synapses, and neurons of the GPe approximately only 2000 synapses. In humans, the number of SNC DA neurons rises to 200,000-420,000, and only approximately 60,000 in the VTA (Björklund & Dunnett, 2007; Moss & Bolam, 2009; Brichta & Greengard, 2014).

Another consideration regarding size and complexity is with regards to striatal volume. Studies have found an approximate 300-fold increase in striatal volume in rats as compared to humans (Oorschot, 1996; Yin et al., 2009; Bolam & Pissadaki, 2012). In contrast, the number of DA neurons only increased by 32-fold from rats to humans (Hardman et al., 2002; Nair-Roberts et al., 2008; Bolam & Pissadaki, 2012). If density and pattern of innervation between species are assumed to be similar, then a single SNC DA neuron in humans should give rise to 10 times the number of synapses than in the rat. In other words, there are an estimated 1-2.4 million synapses in the human striatum, arising from a single SNC DA neuron. In addition, if branching patterns is assumed to be similar between rats and humans, then the average length of an individual SNC DA neuron would be calculated to increase from 47cm in rats to 4.5meters in humans (Bolam & Pissadaki, 2012). This would result in an arborization of incredible complexity.

The SNC and VTA have a very similar quantity of DA neurons in mice. In monkeys and humans however, TH positive neurons in the SNC are present in much higher proportions than in the VTA (Brichta & Greengard, 2014). Due to this, specific species must be taken into account when comparing TH⁺ cell counts across experiments. (*See Table 1*).

Table 1: Number of SNC and VTA DA Neurons in Humans, Monkeys, Rats and Mice

SPECIES	SNC DOPAMINERGIC NEURONS	VTA DOPAMINERGIC NEURONS
HUMAN	200,000-420,000	60,000-65,000
MONKEY	120,000-270,000	110,000
RAT	21,500-25,000	20,000-40,000
MOUSE	8,000-12,000	8,000-12,000

(Brichta & Greengard, 2014)

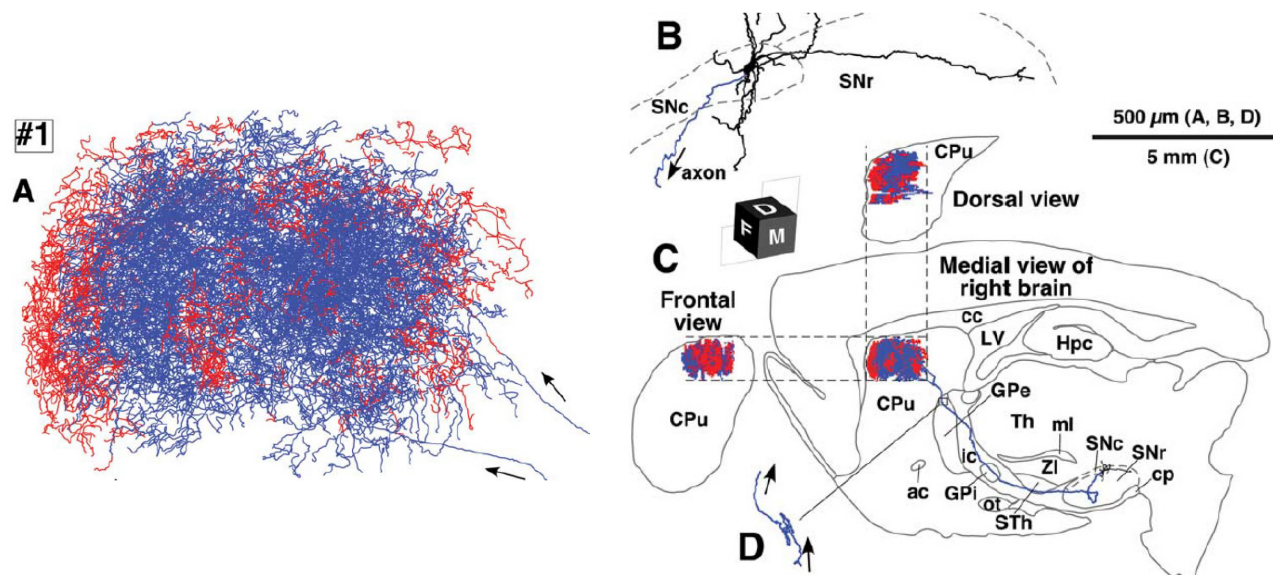
MORPHOLOGICAL CHARACTERISTICS OF SNC DA NEURONS CONTRIBUTE TO VULNERABILITY

As demonstrated, the enormous size, number and complexity SNC DA neurons do not compare to DA neurons of other brain regions. In addition to this, several other morphological characteristics have also been shown to be unique to SNC DA neurons, including: high levels of DAT expression and their large, unmyelinated axonal arbor. The DAT transporter is much more highly expressed in SNC DA neurons than DA neurons of the VTA. This becomes particularly relevant when examining neurotoxins that use DAT for their uptake into the DA cell. For example, the neurotoxins MPTP and 6-OHDA, which utilize DAT, have been found to affect DA neurons of the SNC to a much greater extent than the VTA (Storch, Ludolph & Schwarz, 2004; González-Hernández et al., 2004; Lammel et al., 2008). A study conducted by González-Hernández et al., (2004) demonstrated that both DAT mRNA and protein expression were higher in the SNC than the VTA, and that it was DAT protein expression as opposed to mRNA expression that was attributable to the increased vulnerability of SNC DA neurons.

Studies have also shown that the expression level of DAT is strongly correlated to with the extent of DA cell loss in PD (Storch, Ludolph & Schwarz, 2004).

Perhaps one of the largest underlying factors contributing to SNC DA neuron vulnerability however, is their uniquely large, unmyelinated axonal arbor. The increased vulnerability of neurons possessing these characteristics was discussed in previous sections (Attwell & Laughlin, 2001). It is believed that SNC DA neurons are at a marked disadvantage in terms of resilience because axons of these DA neurons give rise to such a massive axonal field (*Figure 10*) and large number of release sites (Moss & Bolam 2008; Matsuda et al., 2009; Bolam & Pissadaki, 2012). There is substantial recent evidence to support the theory that the unique morphology of SNC DA neurons requires such a huge bioenergetic demand to maintain it and, given that these neurons are already energetically on the edge, they are therefore particularly vulnerable to any type of environmental or genetic stressors that increase their bioenergetic demands (Bolam & Pissadaki, 2012; Pacelli et al., 2015).

Figure 10: Reconstruction of SNC-d DA Neuron



(Figure 10) Reconstruction of the axonal arborization of an SNC-d DA neuron following viral labelling. *Panel A* shows axon fibers in the striatum and *panel B* the dendrites of the same neurons in the SNC. Dorsal and frontal views of intra-striatal axonal arborization were

reconstructed and compared with the medial view. *Red* and *blue* lines in the striatum indicate the axon fibers located in the striosome and matrix compartments, respectively. *Figure reproduced with permission of Matsuda et al., 2009.*

ENERGETIC METABOLISM AND OXIDATIVE STRESS

It has been suggested that PD is a disease of energy metabolism (Wellstead & Cloutier, 2011). The reason for this is that the consequences of having such a large neuronal architecture (*Figure 11*) is that it puts these neurons under exceptionally high energetic demand to maintain their vast array of cellular functions. These functions are essential for proper cell functioning and survival, and include: protein synthesis, cytoskeleton maintenance, axonal transport, maintenance of membrane potential, propagation of action potentials and synaptic transmission (Bolam & Pissadaki, 2012). These aspects of cell function are even more energetically expensive in unmyelinated axons, as is the case with these DA neurons (Attwell & Laughlin, 2001).

Under normal circumstances, these energetic demands can be met by these neurons, however, any factor that increases this demand, such as oxidative stress or mitochondrial dysfunction, may lead these neurons to exceed their energetic supply, putting them in energetic crisis (Bolam & Pissadaki, 2012). Exceeding their energetic supply would put these neurons at a negative energy balance, leading to functional failure and eventually cell death by adding further oxidative stress and mitochondrial dysfunction and leading to an inability to deal with protein turnover, etc. (Bolam & Pissadaki, 2012).

Studies conducted by Pacelli et al. (2015), provided some of the first quantitative evidence that SNC DA neurons have higher basal respiration, higher mitochondrial density, elevated ROS production. They also demonstrated that the axonal arborization in mice is at least 2-fold higher than other DA neurons, conveying an increased vulnerability to certain neurotoxic agents. Their data strongly support the hypothesis that the bioenergetic and unique morphological characteristics of SNC DA neurons underly their increased vulnerability in PD (Pacelli et al, 2015).

Under in vitro conditions, Pacelli et al., (2015) measured and compared the energetic metabolism of SNC DA neurons and less vulnerable DA neurons from the VTA and olfactory bulb (OB). Their results showed that SNC DA neurons not only have a significantly elevated basal mitochondrial respiration compared to other DA neurons, but a smaller respiratory control ratio, elevated ATP content and basal glycolytic flux as well. Their findings suggest that DA neurons are already operating close to the maximum of their mitochondrial energy production capacity in their basal state and that, combined with chronically elevated levels of ROS production and small reserve capacities, it is difficult for these neurons to cope with additional bioenergetic demands, or cellular stresses associated with aging. Additional experimentation demonstrated that, with the use of Sema7, a neuroprotective agent involved in axonal growth, the axonal arborization size of these neurons was reduced, with a corresponding reduction in basal OCR, ROS production, and vulnerability.

As previously stated, PD does not spontaneously develop in rodents, whereas it does in humans. A number of factors should be considered to explain this discrepancy, particularly age. Age is the biggest risk factor for PD, with longevity having significant negative consequences on dopaminergic neurons in humans. Lifespan or longevity however, cannot obviously be compared between humans and rodents. Given that the bioenergetic demand of SNC DA neurons is estimated to be an order of magnitude greater for humans than that for rats, combined with the reduced longevity of rodents, it is likely that these factors account for why humans, and not rats, get PD (Bolam & Pissadaki, 2012).

CALCIUM-MEDIATED CELLULAR STRESS

A common theory regarding the cause of selective vulnerability of SNC neurons is DA toxicity. This theory suggests that the oxidation of cytosolic DA and its metabolites leads to the production of cytotoxic free radicals (Greenamyre & Hastings, 2004; Sulzer, 2007; Surmeier 2010). Recent evidence however, seems to indicate that DA cytotoxicity alone is not enough to explain the loss of DA neurons in the SNC. As discussed, mitochondrial dysfunction is an important factor believed to contribute to oxidative stress and therefore increased vulnerability. Another organelle closely tied to this and the pathogenesis of PD however, is the endoplasmic

reticulum (ER). The ER is an integral component of proteostasis: the production, delivery and degradation of proteins. LB depositions reflect proteostasis deficiency and is accompanied by ER stress (Ryu et al., 2002; Surmeier 2010).

One distinctive physiological phenotype of SNC DA neurons is their ability for pacemaking activity. SNC DA neurons have been shown to be autonomously active, generating action potentials regularly in the absence of synaptic input. This is believed to allow the regions innervated by these neurons, particularly the striatum to maintain DA levels (Grace & Bunney, 1983; Bonci et al., 1998; Surmeier, Guzman & Sanchez-Padilla, 2010). Another related particular attribute of SNC DA neurons is that, unlike other pacemaking neurons that rely only on monovalent cation channels, these neurons also engage rare L-type calcium channels which have distinctive Cav1.3 pore forming subunit. These ion channels allow calcium to enter the cytoplasm, resulting in elevated intracellular concentrations of calcium (Striessnig et al., 2006; Chan et al., 2007; Surmeier, Guzman & Sanchez-Padilla, 2010). These channels are unique in that they open at relatively hyperpolarized potentials, allowing them to contribute to the mechanisms driving autonomous pacemaking.

The sustained engagement of these channels comes at a significant metabolic cost to SNC DA neurons. Calcium is involved in essential cellular processes ranging from enzyme activity regulation to programmed cell death, and in most cells, calcium is kept under tight homeostatic control: channels open rarely and sporadically, and the metabolic costs are manageable. Theoretically, these metabolic costs become much less manageable in SNC DA neurons however, due to the fact that the Cav1.3 calcium channels are open much of the time (Wilson & Callaway, 2000; Surmeier, Guzman & Sanchez-Padilla, 2010). The enormous axonal field of SNC DA neurons and the abnormally low mitochondrial density in their somatodendritic regions is combined with a need for sustained calcium entry. This inevitably leads to a sustained increase in oxidative phosphorylation as well as the production of superoxide and ROS (Liang et al., 2007; Surmeier, Guzman & Sanchez-Padilla, 2010).

The LC and hypothalamic tuberomammillary neurons are also autonomous pacemakers that depend on L-type calcium channels and also demonstrate similar cell loss to the SNC in PD

patients (Williams et al., 1984; Taddese & Bean, 2002; Surmeier, Guzman & Sanchez-Padilla, 2010). These characteristics are not observed in VTA DA neurons, which as mentioned, remain relatively intact in PD. Research to date therefore seems to indicate that calcium-mediated cellular stress is likely an important factor to consider in neurodegeneration.

LIMITATIONS OF CURRENT ANIMAL MODELS OF PD

As discussed, there are currently a number of transgenic mouse models in use for the study of PD. The physiological modifications that are conferred through genetic manipulation has helped researchers to elucidate mechanisms regarding loss or gain of function, which might trigger the disease onset. Whereas there are a number of advantages to using transgenic mouse models, knock-out models have not been able to reproduce the age-dependent loss of DA neurons observed in the human pathology. This could be as a result of the broad effects genetic KO's can have over the entire nervous system; effects that are present from birth. This could also be due to the short lifespan of rodents or, given that their axonal arborization size and bioenergetic demands are so much less than humans, their DA neurons might not have enough intrinsic vulnerability to lead to spontaneous cell loss (Bolam & Pissadaki, 2012; Giguère et al., 2018).

Current KO technologies do present with certain limitations. For example, whereas they can provide information in identifying the roles of certain genes in neural plasticity and development, the absence of these genes since birth could result in gross developmental abnormalities or morbidity (Asada et al., 1997; Heldt & Ressler, 2009). Attempting to determine the involvement of KO genes in developmental defects or compensatory changes can create confounds of whether phenotypic consequences result from lack of normal gene expression in the adult subject or during its development (Heldt & Ressler, 2009). KO methods also not only eliminate the gene activity in the brain, but also in peripheral tissues and the CNS. Also, given that gene function is altered throughout the entire brain, this limits the ability to elucidate their effects on either specific brain regions or neural circuits involved in distinct functions (Heldt &

Ressler, 2009). A more recent solution to these limitations therefore, is the use of conditional manipulation strategies. This conditional approach provides the advantage of more specific temporal and spatial deletion of the gene of interest (Gaveriaux-Ruff, 2007, Heldt & Ressler, 2009).

Perhaps the most important limitation of current rodent models of PD is that they are attempting to mimic a disease of aging that appears in humans over the span of decades, in a species of animal that only has a lifespan of a couple of years. Therefore, in order to mimic PD, the neurodegeneration process in animal models needs to be drastically sped up. This rapid approach causes these models to deviate from human pathology. Also, as discussed, mice also have a much smaller brain than humans, with less dopamine neurons and smaller axonal arborizations. Unfortunately, current animal models do not address these issues. This is very important because, as demonstrated previously, axonal arborization size influences the vulnerability of SNC DA neurons and therefore, presumably, the progression of the disease. One important challenge then is to create an animal model of PD that is more representative of the human pathology by creating adult mice with a much larger axonal arborization and elevated vulnerability.

OBJECTIVES AND HYPOTHESIS

The objective of this project was to develop a novel mouse model of PD that was more representative of the human pathology, by developing adult mice that had fewer DA neurons but larger axonal arbors. The strategy we opted for was to perform unilateral partial lesions to the SNC of P5 mice, leading to the early loss of a large subset of SNC DA neurons and, due to compensatory axonal sprouting, to neurons with a greatly expanded axonal arbour size. Our primary hypothesis was that this should result in SNC DA neurons with elevated vulnerability to secondary stressors.

This project was divided into three parts. The first part of the project involved developing a completely novel mouse model of PD. This involved optimizing a stereotaxic setup that could

accommodate neonatal mice, the development of a cryoanesthesia procedure, and the optimization of placement, volume and dose of the 6-OHDA injection.

The second part of the project required the creation of a partial (~50%) unilateral 6-OHDA lesion of the SNC in neonatal mice. The purpose of this was to attempt to induce compensatory axonal sprouting in residual SNC neurons projecting to the dSTR. This part of the project involved two stereotaxic neurosurgeries: the 6-OHDA lesion at P5 and the injection of an AAV-EYFP virus into either the SNC or VTA at P60 to label the axonal arbour of residual DA neurons. Transgenic Dat-Ires-Cre mice were used to allow for the conditional expression of the EYFP virus in DA neurons. Compensation was confirmed through the use of this virus and confocal microscopy to calculate the volume of the axonal arborization in SNC and VTA neurons.

The third part of the experiment involved measuring the vulnerability of the compensating dopamine neurons. This was accomplished by exposing the surviving DA neurons of the SNC to a secondary neurotoxic stressor: a viral-mediated overexpression of alpha-synuclein. Vulnerability was determined by measuring relative DA neuron survival with stereological counting methods.

In the end, we expected that by creating a mouse model with fewer DA neurons and much larger and more vulnerable axonal arbors, it would be more representative of the human pathology. Very little is known about compensatory mechanisms or how to manipulate them, and this model provides an opportunity to study exactly that.

METHODOLOGY

SUBJECTS

Housing and handling of mice was done with the approval of, and in compliance with the guidelines and regulations of the Comité de déontologie de l'expérimentation sur les animaux (CDEA) of the Université de Montréal. All animals were housed under a 12:12h light/dark cycle and given ad libitum access to food and water. Reproductive couples were transgenic Dat-Ires-Cre (DIC)^{+/+} X C57BL ^{+/+} mice, housed as 2 females and 1 male. Reproductive couples were kept until a maximum of 8 months of age, and all subjects used in this experiment were the DIC^{+/-} pups resulting from this reproduction.

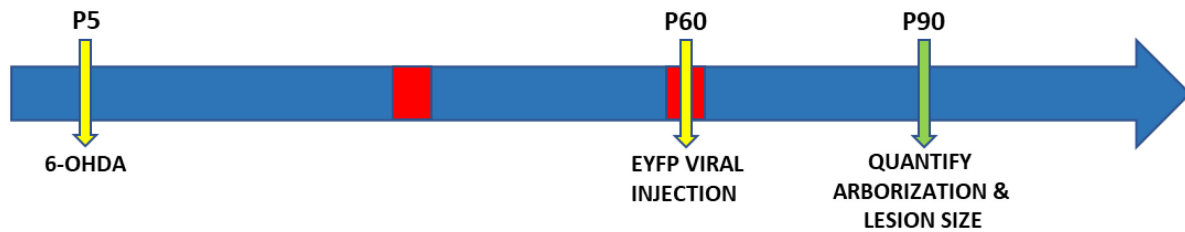
PROJECT TIMELINE

This project was set-up to analyze two primary characteristics of DA neurons following partial lesion to the SNC in neonatal mice: compensation and vulnerability. With regards to compensation, 6-OHDA was unilaterally injected into the SNC of P5 mice and behavioural tests were administered at P30 and again at P60 \pm 3 days. This second set of behavioural tests occurred over several days, just prior to injection of the EYFP virus into the mesencephalon. Mice were then sacrificed at P90 and the tissue was processed to quantify arborization and lesion size.

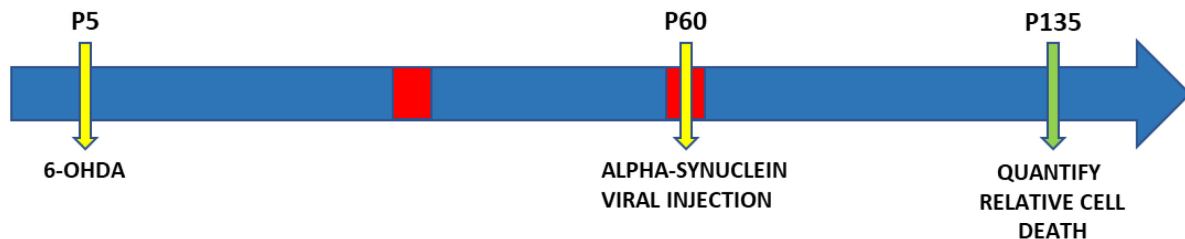
With regards to vulnerability, a separate set of mice received the same 6-OHDA injections and behavioural tests as previously mentioned, however instead of receiving the EYFP viral injection at P60, they received the alpha-synuclein overexpressing virus. These subjects were then sacrificed at P135 and the tissue processed to quantify the relative cell death of DA neurons in the SNC and VTA.

Figure 11: Project Timeline

COMPENSATION:



VULNERABILITY:



(Figure 11) Project timeline for evaluating compensatory processes and their resulting vulnerability. The various surgical procedures at P5 and P60 are indicated in yellow; behavioural testing is indicated in red; the sacrifice of subjects and the subsequent quantification of arborization size and cell death are indicated in green.

NEONATAL SURGERIES

Pups were removed from their home cage 30 minutes prior to surgery and injected with a dose of 2.8mg/mL of desipramine, subcutaneously. Pups were then placed into a latex glove to prevent tissue damage from exposure to the ice and submerged in an ice bath for 3.5 minutes to induce cryoanesthesia. The stereotaxic stand was kept cold with ice packs. The heads of the pups were secured in an acrylic head mold to the stereotaxic apparatus (Digital Lab Standard Stereotaxic for Mice with LED Display, Harvard Apparatus). A small incision was made on the scalp above the lambda suture and a 31-calibre needle attached to the stereotaxic arm was used to puncture the skull at the appropriate coordinates (using lambda as origin). A Hamilton syringe, fitted with a fine glass capillary tip, was connected to an automated pump and used to unilaterally inject either the 6-OHDA solution or the ascorbic acid vehicle solution at a speed of 0.170 μ L/min and volume of 0.5 μ L. Once the injection was complete, the syringe was left in place for 2 minutes to allow for diffusion, then slowly withdrawn from the brain. The incision

was then closed with surgical glue (Surgi-Lock), and the pup was placed in a cage on a heating pad until it had warmed and resumed normal activity. Each surgery was completed within 7-9 minutes. Once all surgeries in the litter were complete, pups were placed back in the home cage.

The volume used for 6-OHDA injection into the neonatal SNC was adapted from current literature on adult mice and determined based on a long process of optimization. For comparison, the volume of 6-OHDA used by Grealish et al., (2010) to induce a lesion to the SNC in adult mice was 1.5µl, whereas the volume used in adult rats to induce a lesion in this target area 3-4µl, as reported by Carman et al., (1991). Given the considerably reduced size of the neonatal mouse brain as compared to the adult, as well as the necessity of limiting the lesion to solely the SNC and not surrounding regions, our preliminary optimization revealed 0.5µl to be a suitable dose for our purposes.

Table 2: Stereotaxic Coordinates of Unilateral 6-OHDA SNC Injection

STEREOTAXIC COORDINATES	UNILATERAL SNC 6OHDA INJECTION
<i>LAMBDA (0,0,0)</i>	<i>VOLUME (0.50µl)</i>
X (Rostrocaudal)	-1.30 mm
Y (Mediolateral)	-0.40 mm
Z (Dorsoventral)	-3.60 mm

INK INJECTIONS

The aim of these neonatal surgeries was to induce a partial (approximate 50%) unilateral lesion of the SNC, while minimizing any cell death to the adjoining VTA. In order to determine the appropriate coordinates for injection in P5 mice, in initial experiments, the same methodology was used as described above, however, black ink was injected at various test

coordinates. The pup was then sacrificed immediately, and brain slices were analyzed for injection coordinates. In this case, ink injections were solely used for the purpose of optimizing the coordinates for neonatal 6-OHDA injection into the SNC. These injections could not be used for quantifying the potential spread/diffusion of the neurotoxin, as the viscosity of the ink did not result in any diffusion, and if it had, it would not have been representative of the diffusion pattern observed with 6-OHDA.

P60 STEREOTAXIC SURGERIES

Lesioned DIC+/- mice underwent a second stereotaxic surgery at P62+/-2. Subjects were injected either bilaterally into the SNC or centrally into the VTA with an AAV-EYFP virus (*See details in Table 3*). The subject was placed in a ventilated chamber and anesthetized with 3% isoflurane until a rate of 1 respiration-per-minute was attained. The subject was then placed on the stereotaxic apparatus and its head secured with ear bars; a mask was placed over its nose to allow for continuous administration of isoflurane at a reduced concentration of ~2% for the remainder of the surgery. Ointment was placed over the eyes of the subject to reduce dryness. Fur on top of the scalp was shaved away and the skin was sterilized with an alternating combination of applied alcohol and iodine. A local anesthetic (1mg/mL Marcaine) was injected subcutaneously under the scalp. After making a 3mm incision along the midline, the point of the surgical drill (diameter 1mm) was zeroed at bregma. Measures were taken at bregma and lambda to ensure a flat skull position. Following this, the drill was re-zeroed at bregma and burr holes were drilled at the appropriate coordinates.

Table 3: Stereotaxic Coordinates of SNC and VTA AAV-EYFP Viral Injections

STEREOTAXIC COORDINATES	BILATERAL SNC EYFP INJECTION	MEDIAL VTA EYFP INJECTION
<i>BREGMA (0,0,0)</i>	<i>VOLUME (0.13μl)</i>	<i>VOLUME (0.05μl)</i>
X (Rostrocaudal)	-3.30 mm	-3.1 mm
Y (Mediolateral)	+/-1.5 mm	0.0 mm
Z (Dorsoventral)	-4.0 mm	-4.5 mm

The surgical drill was then removed from the stereotaxic apparatus and replaced by a 10 μ l Hamilton syringe fitted with a fine glass capillary tip. With the use of binoculars, the micropipette was zeroed at Bregma and moved to the appropriate coordinates. To ensure proper functioning and no blockage, a small quantity of the virus was ejected before slowly lowering the pipette through the burr hole to the required Z-coordinates. The virus was then injected at a rate of 0.250 μ l/min and the micropipette was left in place for an additional 5 minutes to allow for diffusion. The syringe was then slowly withdrawn from the brain and the wound was cleaned and sutured. A subcutaneous injection of Caprofen and saline was administered post-operatively at a dose of 5mg/kg and concentration of 50mg/ml to provide rehydration and analgesia.

The same surgical protocol was followed for injecting the alpha-synuclein overexpressing virus, with modification to the volume and coordinates. The aim was to bilaterally infect both the SNC and VTA equally and in their entirety.

Table 4: Stereotaxic Coordinates of Bilateral SNC & VTA Injections of the Alpha-Synuclein Overexpressing Virus

STEREOTAXIC COORDINATES	BILATERAL SNC/VTA VIRAL INJECTION
<i>BREGMA (0,0,0)</i>	<i>VOLUME (1.50μl)</i>
X (Rostrocaudal)	+/-1.0 mm
Y (Mediolateral)	-3.10 mm
Z (Dorsoventral)	-4.30 mm

It should be noted that there is a large variation between the volume injected for each type of drug in this experiment. This is due to the varying circumstances and goals of each of these injections. For example, 0.5ul of 6-OHDA was injected into the SNC of P5 mice, whereas 0.13ul of AAV-EYFP virus was injected into the SN of P60 mice. The first factor to consider is that each of these solutions has varying diffusion properties based on their specific concentrations, viscosities, etc. Whereas specific measures of these diffusion properties were not quantified in this experiment, optimization of these protocols our observations determined that a larger volume of 6-OHDA was needed in neonatal mice than the viral injection in adult mice. This indicates that the viral solutions likely have a much more wide-ranging diffusion pattern than the 6-OHDA solution. In addition, the differing concentrations of these solutions also need to be taken into consideration, as it also appears to require a higher concentration of 6-OHDA to degenerate SNC DA neurons than it does to infect these neurons with an EYFP-expressing virus. Lastly, a final consideration should be made with regards to the anatomy and morphology of neonatal and adult mouse brains. It is possible that based on these characteristics of the developing mouse brain, solutions may not be able to diffuse as easily or as far as they do in adult brains, which could again account for some of the differences required in the volumes injected.

DRUGS

6-HYDROXYDOPAMINE (6-OHDA)

6-hydroxydopamine hydrobromide was dissolved in 0.02% ascorbic acid solution at a concentration of 2mg/mL and administered in a volume of 0.50 μ l. The drug solution was prepared just prior to use, kept at a cold temperature and protected from light.

DESIPRAMINE

The desipramine solution was prepared at a concentration of 2.8mg/kg and was administered subcutaneously to the neck of pups 30 minutes prior to 6-OHDA surgery in a volume of 300 μ l.

AAV-EYFP VIRUS

The fluorescent virus used was rAAV2/Ef1a-DIO-EYFP (UNC GTC Vector Core, lot AV4842d, 4 x 10¹² virions/ml), diluted 1:4 in a 0.9% NaCl solution. Solution was prepared just prior to use and administered in a volume of 0.13 μ l in the SNC and 0.05 μ l in the VTA.

ALPHA-SYNUCLEIN VIRUS

The alpha-synuclein overexpressing virus was an AAV2-CBA-alpha-synuclein virus (UNC Vector Core) and diluted in a 1:4 0.9% NaCl solution. Solution was prepared just prior to use.

PERFUSION

At selected time points, mice were terminally anesthetized with an intraperitoneal injection of 100mg/kg pentobarbital (7mg/mL) and intracardially perfused with 100mL of ice-cold phosphate buffer saline (PBS) solution and 100mL 4% paraformaldehyde (PFA) solution. Brains were extracted, incubated in PFA for 48 hours, and followed by a 30% sucrose solution for 48 hours to prepare for cryostat slicing.

CRYOSTAT

Once removed from the sucrose solution, brains were frozen and sliced at 40 μ m thickness in the coronal plane using a cryostat. Sections were collected in six series and stored at 4 degrees in antifreeze (phosphate buffer containing 30% ethylene glycol and 30% glycerol) until free-floating immunohistochemistry was performed.

IMMUNOHISTOCHEMISTRY FOR IMMUNOFLUORESCENCE

One of six series of brain sections prepared with the cryostat was randomly selected from each brain for processing. Triple immunofluorescent staining was performed under uniform conditions on free-floating sections. Striatal sections were stained for use in confocal microscopy to quantify axonal arborization size, whereas mesencephalic sections were stained for verification and quantification of the EYFP viral infection. Primary and secondary antibodies were combined with a Goat serum solution containing: PBS, 0.5% bovine serum, 0.3% Triton X-100, 0.02% sodium azide and 5% goat serum. Sections were first triple rinsed (3x10min) with 0.01M PBS solution and placed into a block solution (Goat serum solution enriched with bovine serum) to permeabilize and block nonspecific binding of the primary antibody. Following a 1-hour incubation period, sections were then transferred directly into the primary antibody solution: tyrosine hydroxylase (TH, rabbit polyclonal, 1:2000), green fluorescent protein (GFP, chicken polyclonal, 1:2000), and dopamine transporter (DAT, rat polyclonal, 1:2000) and agitated at room temperature for 24 hours. Following this incubation period, sections were triple rinsed again and incubated in the dark, on an agitator, in a secondary antibody solution for two hours: Alexa 546 Rabbit (1:500), Alexa 488 Chicken (1:500), Alexa 647 Rat (1:500). After a final triple rinse, sections were mounted and cover-slipped with Fluoromount.

IMMUNOSTAINING FOR STEREOLOGY

3,3'-Diaminobenzidine (DAB) immunohistochemistry was performed under uniform conditions on free-floating mesencephalic sections, using an antibody raised against tyrosine hydroxylase (TH, rabbit polyclonal, 1:1000) for the purpose of stereological counting of TH-positive neurons in the SNC and VTA. One of six wells were randomly selected for processing. All antibodies were combined with a solution containing 0.01M PBS and 0.3% Triton. Sections were pretreated with 0.9% hydrogen peroxide for 10 minutes to quench endogenous peroxidase activity, triple rinsed (3x10 minutes) with PBS and incubated for 48 hours at 4 degrees in the primary antibody solution. Following this incubation period, sections were triple rinsed again, before undergoing a 12-hour incubation period at 4 degrees in a secondary antisera solution (Biotin-SP Goat Anti-Rabbit; 1:200). Following another triple rinse, sections were incubated in a tertiary solution (Streptavidin Horseradish Peroxidase, 1:200) for 3 hours at room temperature. Sections underwent a final triple rinse in PBS solution, followed by a 1-minute rinse in a 0.1M acetate buffer solution. TH immunoreactivity was visualized using 3'3-diaminobenzidine. Sections were mounted and left to dry for 72 hours before undergoing a Nissl and defatting process. Slides were then cover-slipped with Permount and allowed to dry. The aim of the Nissl and defatting process is to improve the optical characteristics of tissue sections and, using cresyl violet solution, adds a violet/blue cytoplasmic RER stain to all cells to help identify non-neuronal cells and TH-negative neurons.

QUANTIFYING AXONAL ARBORIZATION SIZE OF DOPAMINE NEURONS

CONFOCAL MICROSCOPY

To quantify the size of the axonal arborization and determine whether compensation occurred in surviving DA neurons following partial cell loss in the SNC, striatal sections were triple stained with TH/GFP/DAT immunofluorescence (as previously described) and imaged using a confocal microscope (Model FV1000 Olympus, utilizing software FV-10 ASW 3.1) at 20x magnification. Up to 4 images were taken in the dSTR per hemisphere, and 1-3 images in the vSTR. Each section was matched with its corresponding Bregma coordinates using the

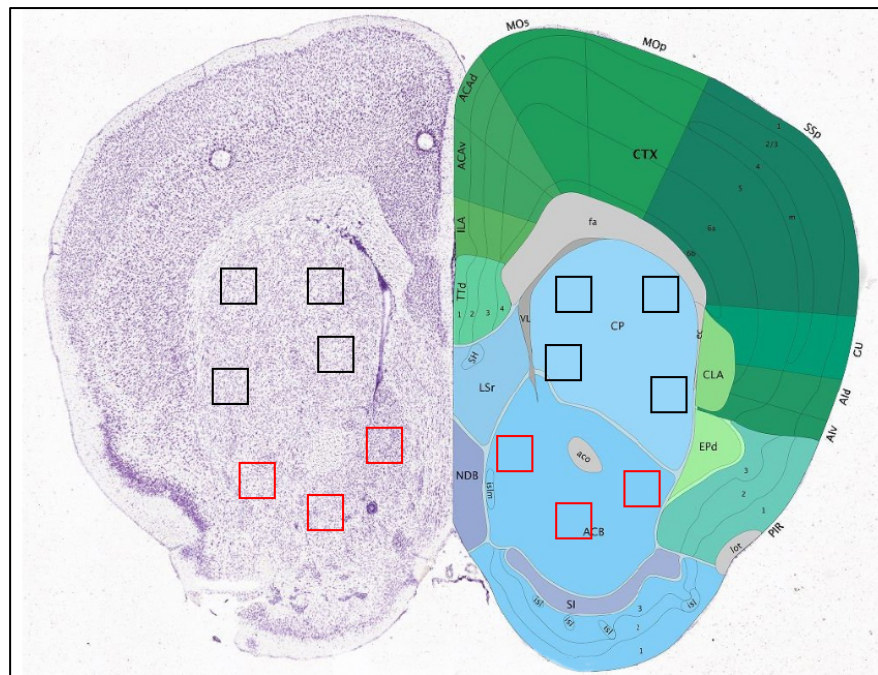
Mouse Atlas (Paxinos & Franklin, 2012), and the area of both the dorsal and ventral striatum was calculated for each of these bregma coordinates. Images were thresholded for GFP intensity and the percent area covered by the GFP signal was calculated in Fiji ImageJ. Fluorescent staining on mesencephalic sections allowed for manual quantification of the number of EYFP-infected cells in the SNC and VTA for each hemisphere.

The average percent area covered by GFP signal was calculated for the dorsal and ventral striatum of each slice. This average percent area was then divided by the number of EYFP-infected neurons of that hemisphere to calculate the percent area covered by infected neurons. This value was then multiplied by the area of the striatum in that respective slice to calculate the total area covered by infected neurons. The axonal length was compared between lesioned and non-lesioned hemispheres. Photomultiplier voltage and laser excitation settings were kept constant for all brains within the same immunostaining and determined based on obtaining the highest possible intensity without saturation.

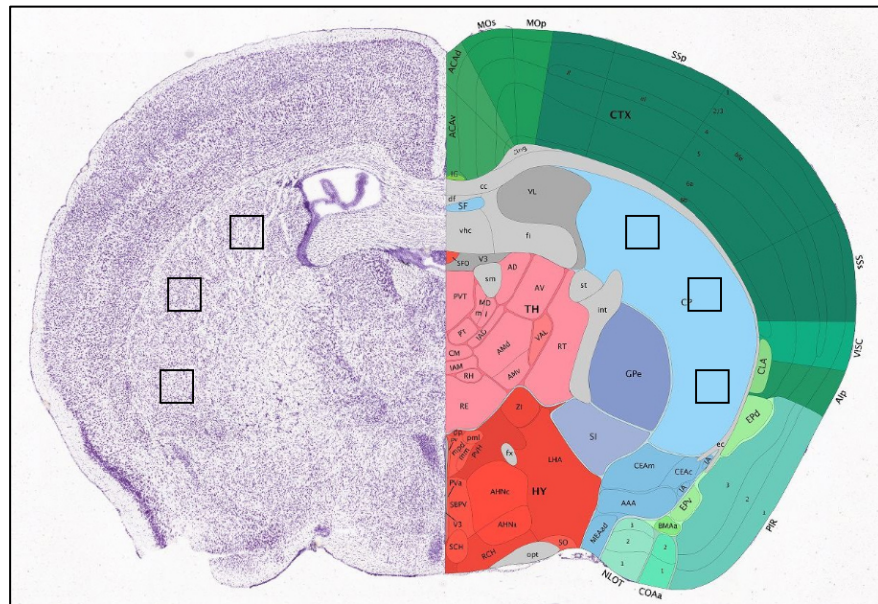
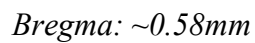
Figure 12: Representation of sites sampled for confocal microscopy

ROSTRAL

Bregma: ~1.42mm



Bregma: ~0.98mm



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STEREOLOGY

Unbiased stereological analysis was conducted using the optical fractionator probe to estimate the number of surviving TH⁺ cells in the SNC and VTA. The borders of the SNC and VTA were delineated along the rostro-caudal axis using a low power objective lens (10X). The sections used for counting covered the entire rostral-caudal range of the SNC and VTA, typically yielding 6-7 sections in a 1:6 series. Stereological counts were made using a 100X oil-immersion objective on a microscope with a motorized stage. The sampling software, Stereo Investigator (MBF Bioscience), used parameters of 40 μ m thickness per slice, 10 μ m depth (optical fractionator), a counting frame of 50 μ m x 50 μ m, and a sample size of 150 μ m x 150 μ m. Coefficient of error attributed to the sampling was calculated as the Gunderson coefficient. Stereological counts of the RLi, CLi and IF were not included as part of the VTA.

BEHAVIOURAL TESTING

Prior to behavioural testing, mice were moved to a room separate from the colony and separated into individual cages 30 minutes prior. Subjects were tested between P30 and P35 and a subset of subjects were tested between P55 and P60 for age comparison, before undergoing the P60 AAV-EYFP surgery.

ROTAROD

The instrument consists of a rotating spindle (5cm diameter), a power source for turning the spindle, and levers placed below the spindle where the mice can fall (LE8205, BioSeb). All mice were pre-trained on the rotarod for two consecutive days to reach a stable performance, then tested on day three. On the first day, mice were placed on the rotarod, rotating at a constant speed of 4rpm. They remained on the rotarod until either one minute had passed, or up to a maximum of 3 attempts at placing them on rod. On the second day, mice were trained on an accelerated rotation setting: 4-40rpm over a ten-minute period. The latency to fall was recorded. This training was repeated 3 times with approximately 5 minutes rest between trials. The same parameters were used on the test day (day 3).

GRIP STRENGTH TEST

For the grip test, mice were held firmly by the tail above the grid, and slowly lowered until their forepaws grasped the middle of the grid. Subjects were then lowered to a horizontal position and pulled following the axis of the sensor until they released their grasp on the grid. The maximum force exhibited by the subject was recorded. This test was repeated 3 times, with approximately 2 minutes of rest between each test. The body weight of the mice was recorded and the mean of the three trials was calculated.

CYLINDER TEST

Mice were individually placed in a 4L cylinder beaker, without time to acclimate. Mirrors were used to allow 360-degree observation, and subject behaviour was live-scored for a ten-minute period. The number of rotations ipsilateral and contralateral to the lesion were measured. Preferential forepaw use was also simultaneously measured as the number of weight-bearing wall touches during rearing. Forepaw use ipsilateral and contralateral to the lesion were recorded, as well as the use of both paws simultaneously.

Apomorphine-induced rotation was also measured, as it reflects the hypersensitivity of the lesioned striatum. Apomorphine was administered subcutaneously in a subset of subjects, at a dose of 0.5mg/kg, five minutes prior to testing. Subjects were then removed from their individual cages and placed in the cylinder for live-scoring of rotations for a ten-minute period.

LOCOMOTION

Locomotor behavior was recorded using an infrared actimeter (Superflex sensor version 4.6, Omnitech) with Fusion software (v5.6 Superflex Edition). Total distance, rest time, movement time, stereotypy time, ambulatory activity and ambulatory time was measured. A chamber partition was used to measure two mice at a time. Subjects were not given time to acclimate and spent a total of 30 minutes in the chamber.

STATISTICAL ANALYSIS

Statistical analysis was performed using the GraphPad Prism 7.0 software package. Results are expressed as means \pm standard error of the mean (SEM). The student T test and 1-way anova multiple comparisons were used. Significance was defined as: $P < 0.05$ *; $P < 0.01$ **; $P < 0.001$ ***

RESULTS

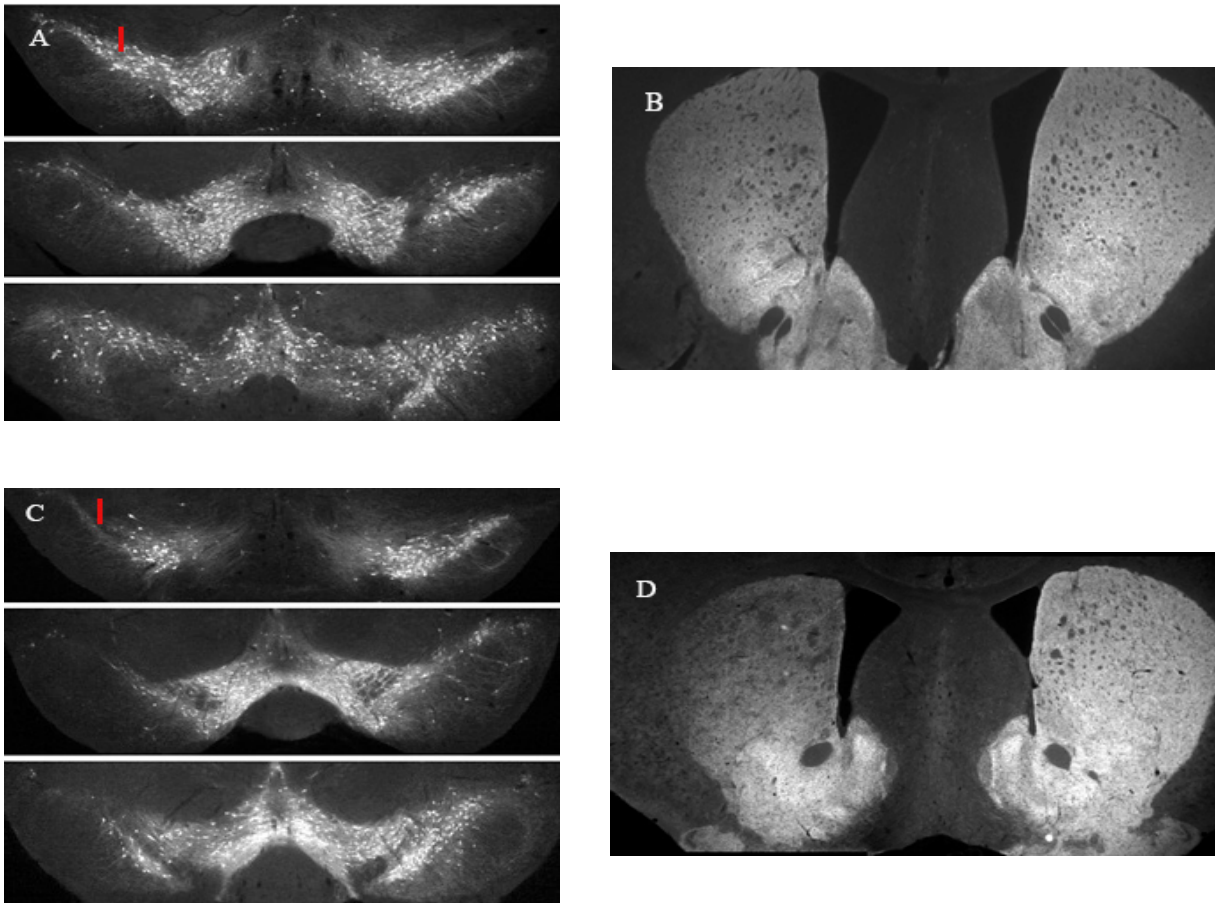
Given the lack of current research on neonatal stereotaxic surgeries, this project started out by optimizing a protocol that would allow us to create partial (~50%) unilateral lesions to the SNC of neonatal mice. Many factors had to be established and optimized for this protocol: time duration of cryoanesthesia for optimal survival, stereotaxic coordinates for placement of injection to limit the lesion selectively to the SNC, and the concentration and volume of 6-OHDA had to be determined to induce a partial lesion in our target range. In addition, given the lack of commercially available stereotaxic equipment for neonatal mice, our stereotaxic setup had to be modified.

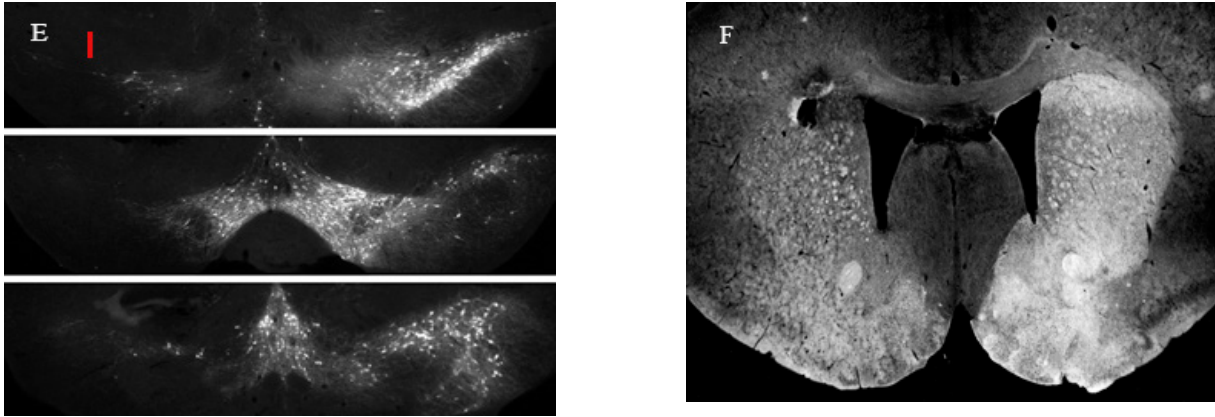
Once the stereotaxic coordinates were determined using ink injections, various doses of 6-OHDA·HBr were tested for lesion size: 0.5mg/mL, 2mg/mL and 6mg/mL. The goal was to identify the dose of 6-OHDA required to induce approximately 50% cell death in the SNC of mice. The neurotoxin was injected at P5 and brains were extracted and processed at P15 to estimate the lesion size. Processing of these brains involved cryostat slicing at 40um thickness, with slices being serially distributed among 6 wells. For stereological counting, one of the six wells was chosen at random for DAB staining and quantification of dopaminergic cell bodies. The dose of [0.5mg/mL] (*Figure 11A&B*) induced a small partial lesion, estimated at approximately 20%. The dose of 2mg/mL (*Figure 11C&D*) however, induced a 40-60% lesion, which was within the target range. The dose of [6mg/mL] (*Figure 11E & F*) which was the initial testing dose, induced a near-complete lesion of the SNC, and significant cell death of the adjoining VTA. Preliminary results indicated that the size and position of the [2mg/mL] 6-OHDA lesion resulted in minimal loss of TH⁺ neurons in the VTA and dopaminergic cell death in the SNC within our target range. This dose was chosen to use for the duration of the experiment.

STEREOLOGICAL COUNTS OF TH+ NEURONS IN THE SNC AND VTA

At a dose of 2mg/mL 6-OHDA·Br and volume of 0.5ul injected into the SNC of P5 DIC+/- pups, the lesion size ranged from 5-96%. Stereological counting revealed that 78% (32/41 brains) were within the range of a 50-80% lesion size. Slightly higher than our preliminary results indicated, the final calculated average lesion size was 60% for the SNC and 30% for the VTA.

Figure 13: TH-immunofluorescent staining of mesencephalon and striatum following 6-OHDA injection

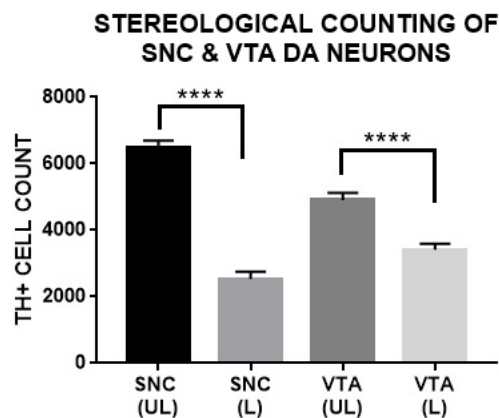




(Figure 13) TH-immunofluorescent staining of mesencephalon and striatum following 6-OHDA test doses. Three different doses of 6-OHDA were tested to optimize the protocol. 3 different slices of each mesencephalon showing various levels of cell death at each dose are presented here. *Red bars* indicate the approximate injection site. Striatal slices demonstrate level of TH-innervation following lesion. (A) [0.5mg/mL] 6-OHDA injection to the left SNC and accompanying striatal section (B). Level of cell death was estimated at ~20% for this dose. (C) [2mg/mL] 6-OHDA injection to the left SNC (C) with accompanying striatal section (D). This dose of neurotoxin induced an approximate 50-60% lesion in the SNC. (E) [6mg/mL] 6-OHDA injection with accompanying striatal section (F). This dose of 6-OHDA induced a near complete lesion of the SNC and significant cell death in the adjacent VTA.

Of the 41 brains, the average TH+ stereological count was 6478 in the SNC of the unlesioned (UL) hemisphere, compared to an average of 2527 TH+ cells in the SNC of the lesioned (L) hemisphere. The mean TH+ cell count of the VTA in the unlesioned hemisphere was 4906, compared to 3416 in the lesioned hemisphere. Using Sidak's multiple comparison test, the differences in TH+ counts of the unlesioned and lesioned SNC, as well as the unlesioned and lesioned VTA, were both statistically significant ($p < 0.0001$) (Figure 14).

Figure 14: Stereological counts of SNC & VTA DA neurons



(Figure 14) Stereological counts of TH+ neurons of the SNC & VTA. Average TH+ cell count (n=40) in the unlesioned SNC was 6478, as compared to 2527 in the lesioned hemisphere ($p<0.0001$). Average TH+ cell count of the VTA in the unlesioned hemisphere was 4906, as compared to 3416 in the lesioned hemisphere ($p<0.0001$). This results in an approximate 60% lesion to SNC DA neurons and ~30% in the VTA at a dose of [2mg/mL] 6-OHDA.

Whereas the target lesion size was approximately 50% for the SNC, the final analysis revealed 60% TH+ cell death in the SNC and 30% TH+ cell death in the VTA. The objective of creating a partial unilateral lesion in the SNC of neonatal mice was achieved.

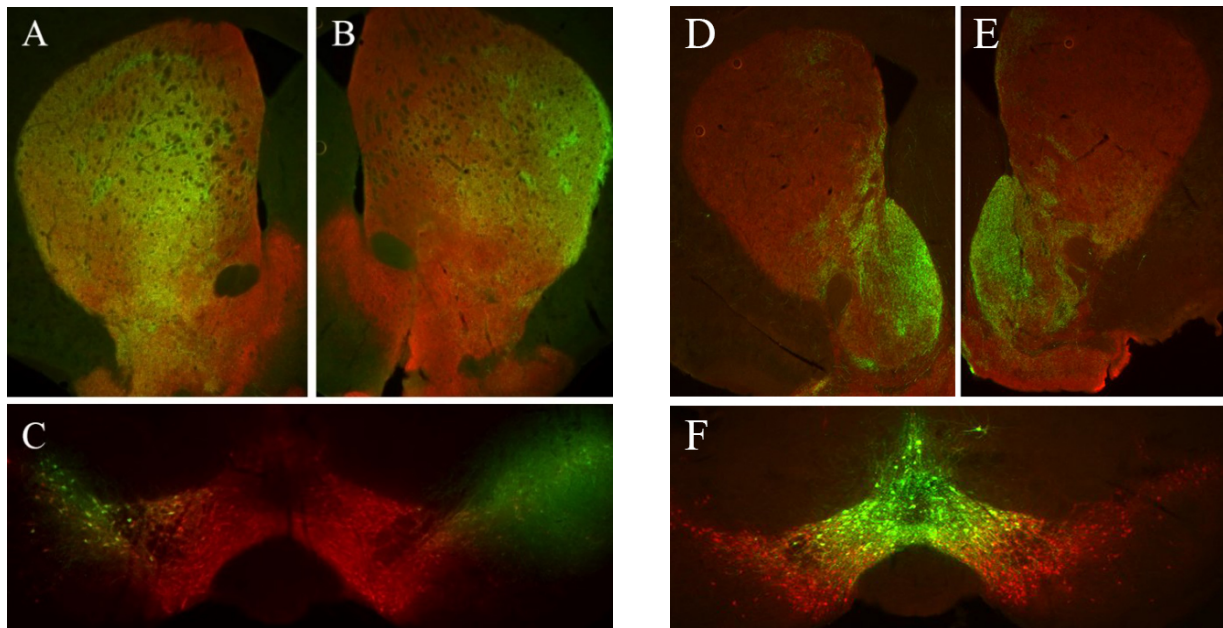
INCREASE IN AXONAL ARBORIZATION SIZE FOLLOWING PARTIAL LESION

Having identified a dose of 6-OHDA sufficient to induce the loss of approximately 60% of SNC DA neurons, we next sought to estimate the axonal arborisation size of residual SNC and VTA DA neurons. To accomplish this, a conditional EYFP virus was injected either centrally into the VTA or bilaterally in the SNC at P60. Subjects were sacrificed at P90 to process the brains for TH/EYFP immunofluorescence.

Figure 15 presents samples of the 4X images of double stained TH/GFP tissue taken from the striatum (*A-B, D-E*) and mesencephalon (*C & F*) of lesioned P90 mice, 30 days post-

viral injection. Images on the left demonstrate the bilateral viral infection to the SNC and corresponding dSTR (*A-C*), whereas images on the right are of a centrally infected VTA and corresponding vSTR (*D-F*). The lesion in these images is present in the left hemispheres, though, as can be seen with the TH staining (*TH in red, GFP in green*) in the striatal sections, induced cell death targeted to the dorsal and lateral tiers of the SNC does not necessarily correspond with denervation to a specific region subregion of the striatum. Instead, the TH denervation of the dSTR was relatively uniform. EYFP-infected cell bodies in mesencephalic slices were manually counted at 10X magnification for the SNC and VTA and used for the calculation of axonal arbor size.

Figure 15: Immunofluorescent images of the TH/GFP signal in striatal and mesencephalic slices of lesioned mice



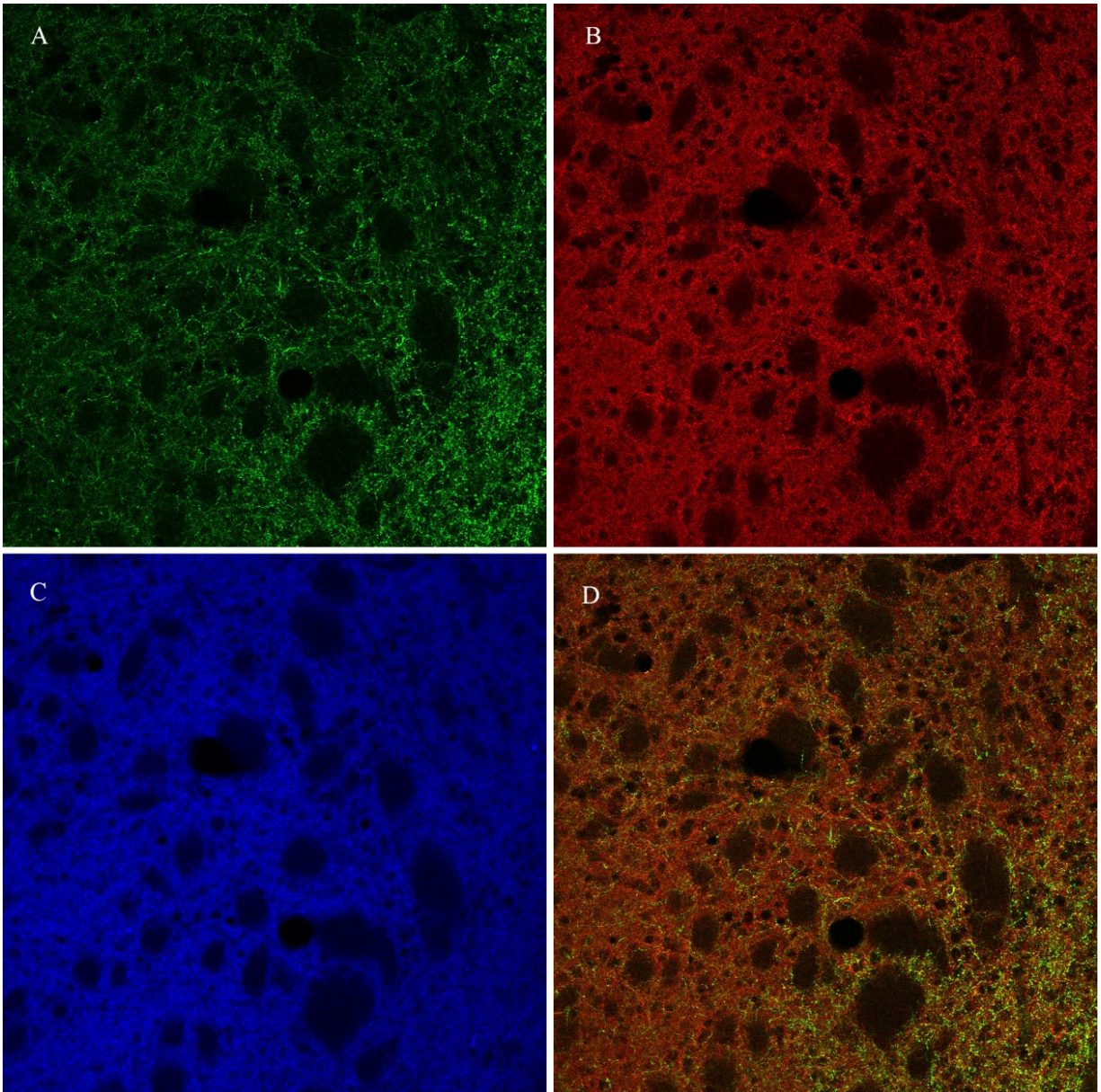
(Figure 15) 4X images of TH and EYFP expression in the left and right striatal hemispheres (*A & B*). The EYFP signal is represented in green and the TH signal in red. The signal obtained after bilateral infection of the SNC is shown in *Figure C*. *Figures D & E* show bilateral infection of the ventral striatum resulting from a centralized injection into the VTA (*Figure F*). The number of EYFP-infected cells in the mesencephalon was counted manually under fluorescence and used in conjunction with the percentage of surface area covered in 20X confocal images of the striatum to calculate the volume of axonal arbors.

Once the number of EYFP-infected cells were manually counted in the SNC and VTA of each subject, striatal sections were then imaged using confocal microscopy and used to quantify axonal arborization size. Figures 16 & 17 below are examples of these 20X confocal images; GFP (*A*), TH (*B*) and DAT (*C*) signals were captured simultaneously. Steps were taken to ensure the signals were not saturated. The DAT signal was primarily used to help distinguish the borders between dSTR and vSTR regions. The TH and GFP signal overlap is shown in *panel D*. Figure 16 was an image taken from the upper/outer region of the unlesioned dSTR of an SNC EYFP-infected brain. Figure 17 depicts the vSTR in the unlesioned hemisphere of a VTA EYFP-infected brain. Depending on the size of the striatal area, images captured in the dSTR were sampled from 2-4 regions per hemisphere, and 1-3 images per hemisphere were captured in the vSTR. All regions sampled were mutually exclusive (there was no overlap). Figures 16 & 17 are representative of brains with a large number of EYFP-infected cells in the mesencephalon. Due to high variability, some brains resulted in a much lower number of infected cells and corresponding striatal infection. To avoid bias, the same striatal regions were sampled in each brain regardless of the level of infection.

These 20X images were collected and separated into dorsal and ventral striatal regions, as well as unlesioned and lesioned hemispheres. The GFP signal was then thresholded to eliminate noise and the area of each signal was then measured using ImageJ. This signal area, combined with the manual counts of either SNC or VTA EYFP-infected neurons in the mesencephalon, was used to calculate the size of the axonal arbor of the infected DA neurons. A comparison between unlesioned and lesioned hemispheres was then made to determine if a difference in axonal arborization size was detectable.

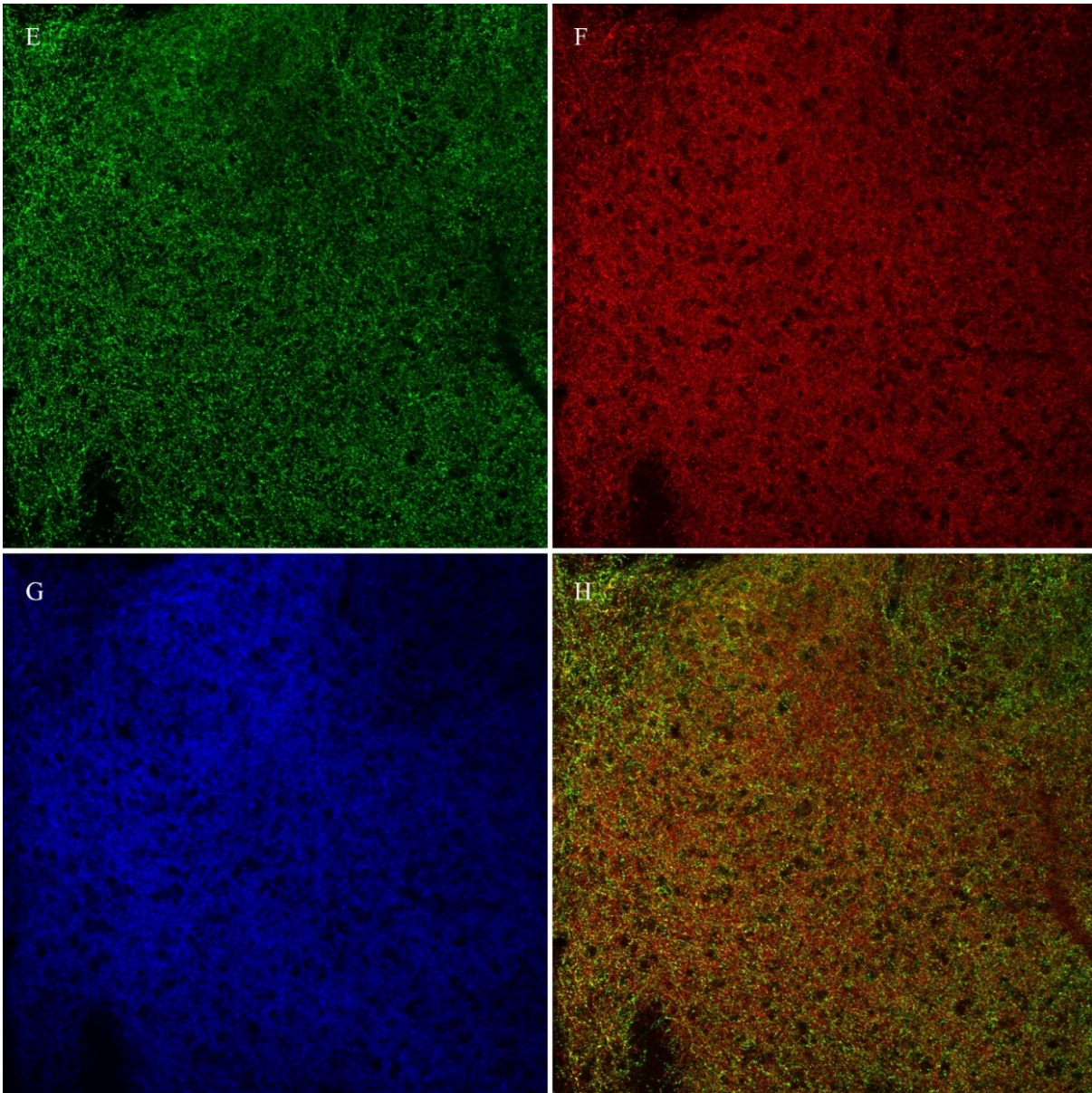
Overall, stereotaxic injection of the EYFP-expressing virus into either the SNC or VTA was successful, and used for calculation of the change in axonal arborization size of dopaminergic neurons between lesioned and unlesioned hemispheres.

Figure 16: 20X confocal images of the EYFP-infected dSTR



(Figure 16) The figures above are examples of 20x confocal images taken of triple stained dSTR tissue in the unlesioned hemisphere of P90 mice having undergone both 6-OHDA and AAV-EYFP treatments. Fluorescent immunohistochemistry was performed to visualize and quantify the GFP signal (*A*), TH signal (*B*), DAT signal (*C*) and a merge of both GFP and TH signal (*D*).

Figure 17: 20X Confocal images of the EYFP-infected vSTR



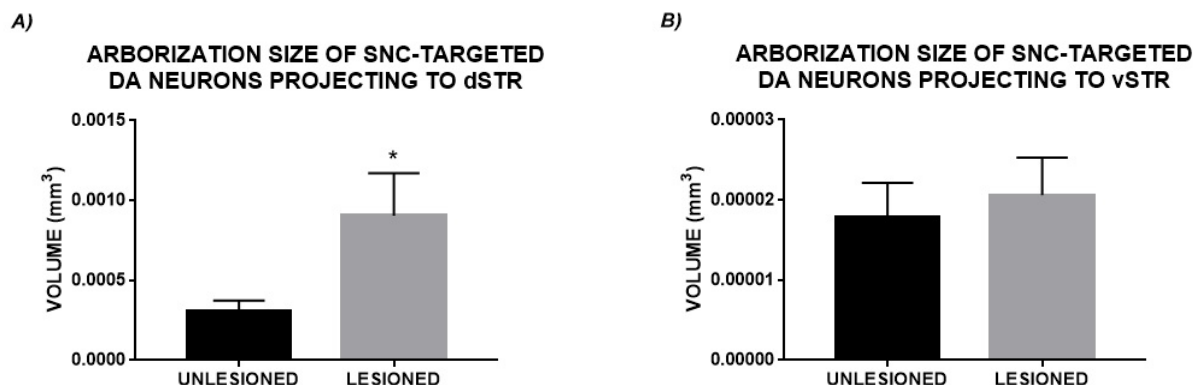
(Figure 17) The figures above are examples of 20x confocal images taken of triple stained vSTR tissue in the unlesioned hemisphere of P90 mice having undergone both 6-OHDA and AAV-EYFP treatments. Fluorescent immunohistochemistry was performed to visualize and quantify the GFP signal (*E*), TH signal (*F*), DAT signal (*G*) and a merge of both GFP and TH signal (*H*).

In the following analysis it is important to note that descriptions of SNC and VTA neurons projecting to dSTR or vSTR are in fact SNC-targeted neurons and VTA-targeted neurons. In this analysis, the exclusion criteria that was set for cross-contamination of EYFP infection into regions other than the targeted region (i.e. SNC or VTA) was 25%. In other words, brains were accepted into analysis if a minimum of 75% of the total number of infected neurons were in the target region. The reason for this level of exclusion is explained in further detail in the discussion.

To establish that compensatory sprouting did occur in the surviving DA neurons, the volume of axonal arborization was calculated in EYFP-infected DA neurons projecting from the SNC to the dSTR in the unlesioned and lesioned hemispheres (*Figure 18A*). Dopaminergic neurons projecting from the SNC to the dSTR in the unlesioned hemisphere had a mean volume of 0.00030mm^3 ($n=15$), whereas DA neurons projecting from the SNC to the dSTR had a mean calculated volume of 0.00090mm^3 ($n=15$) in the lesioned hemisphere. This supports our hypothesis that a partial lesion can induce compensatory axonal sprouting in surviving DA neurons. We found here that the volume of the axonal arbor of compensating SNC DA neurons increased by approximately 2.1-fold ($p=0.0384$) as compared to SNC DA neurons of the unlesioned side.

Analysis of SNC EYFP-infected DA neurons projecting to the vSTR (*Figure 18B*) revealed a calculated mean volume of $1.789\text{e-}005\text{mm}^3$ ($n=17$) in the unlesioned hemisphere, as compared to $2.06\text{e-}005\text{mm}^3$ in the lesioned hemisphere ($n=11$). The unpaired t test determined there was no statistically significant difference between these two groups ($p=0.678$). Given that SNC neurons do not generally project to the vSTR, these numbers are likely a result of slight contamination of the SNC/VTA infection. Due to the selected level of threshold for exclusion, and therefore cross-contamination, axonal arborization size of targeted SNC-infected neurons projecting to the vSTR are not included for further statistical analysis. The authors also found no literature supporting the existence of SNC DA neurons projecting to the vSTR nor any potential significance of it.

Figure 18: Axonal arborization size of SNC-targeted DA neurons projecting to the dSTR and vSTR



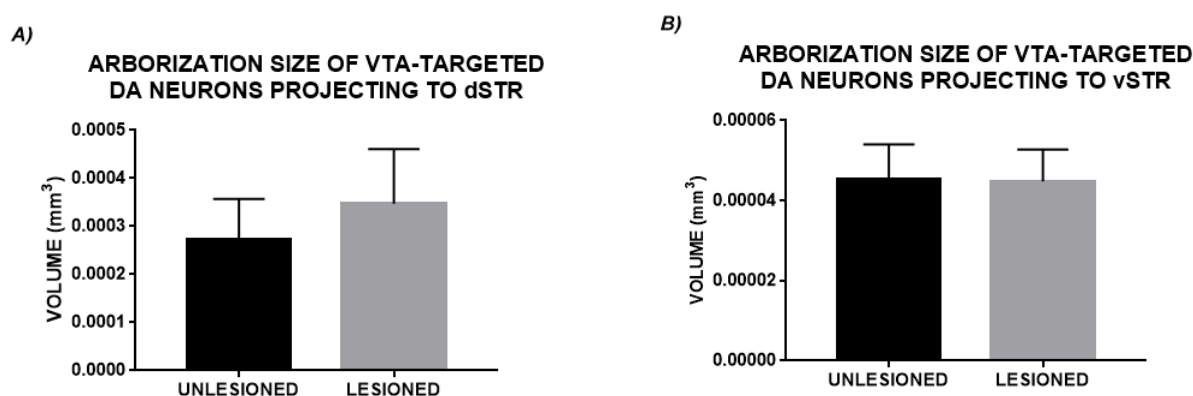
(Figure 18) Comparison of axonal arborization size of SNC-targeted DA neurons projecting to the dSTR and vSTR in unlesioned and lesioned hemispheres. (A) Mean volume of SNC-targeted DA neurons projecting to the dSTR in the unlesioned hemisphere was 0.0003345mm^3 (n=15) as compared to 0.0007173mm^3 in the lesioned hemisphere (n=15). This indicates a 2.1-fold increase in axonal arborization size as a result of compensatory sprouting in the residual SNC DA neurons of the lesioned hemisphere. (B) Mean volume of SNC-targeted DA neurons projecting to the vSTR was $1.788\text{e-}005\text{mm}^3$ in the unlesioned hemisphere (n=17) as compared to $2.06\text{e-}005\text{mm}^3$ in the lesioned hemisphere (n=11).

Research has suggested that compensatory sprouting can occur in VTA DA neurons in response to lesions of the SNC (Ho et al., 1998; Schneidman et al., 2003; Arkadir, 2014). Therefore, the same analysis as described above was performed for targeted VTA-infected neurons. As can be seen in Figure 19, there was no significant difference detected between unlesioned and lesioned hemispheres in the mean volume of the axonal arbor of VTA-infected DA neurons that projected to either the dSTR or vSTR. EYFP-infected DA neurons projecting from the VTA to the dSTR had a calculated average volume of 0.000273mm^3 (n=9) in the unlesioned hemisphere, whereas VTA-targeted neurons projecting to the dSTR in the lesioned hemisphere had an average volume of 0.000346mm^3 (n=12; p=0.624). It could be argued a slight tendency is observed here, however not statistically significant. It should be noted here that the majority of VTA neurons are expected to project to the vSTR, however, as a result of previously discussed compensatory mechanisms, some VTA neurons could have expanded their

projections into the dSTR. The possibility of some cross-contamination from DA neurons of the SNC also exists in this case.

As expected, no significant difference was detected in VTA-infected DA neurons projecting from the VTA to the vSTR, with a calculated mean volume of $4.52 \times 10^{-5} \text{mm}^3$ in the unlesioned hemisphere and $4.47 \times 10^{-5} \text{mm}^3$ in the lesioned hemisphere ($p=0.967$).

Figure 19: Axonal arborization size of VTA-targeted DA neurons projecting to the dSTR and vSTR



(Figure 19) Comparison of axonal arborization size of VTA-targeted neurons projecting to the dSTR and vSTR in unlesioned and lesioned hemispheres. (A) The mean volume of VTA neurons projecting to the dSTR in the unlesioned hemisphere was 0.000273mm^3 ($n=9$) as compared to 0.000346mm^3 ($n=12$) in the lesioned hemisphere. Unpaired t-test analysis revealed there was not a significant difference between these hemispheres ($p=0.6242$). (B) The mean volume of axonal arborization size of VTA neurons projecting to the vSTR is $4.52 \times 10^{-5} \text{mm}^3$ in the unlesioned hemisphere ($n=13$) as compared to $4.47 \times 10^{-5} \text{mm}^3$ in the lesioned hemisphere ($n=11$). There was no statistically significant difference between hemispheres ($p=0.967$).

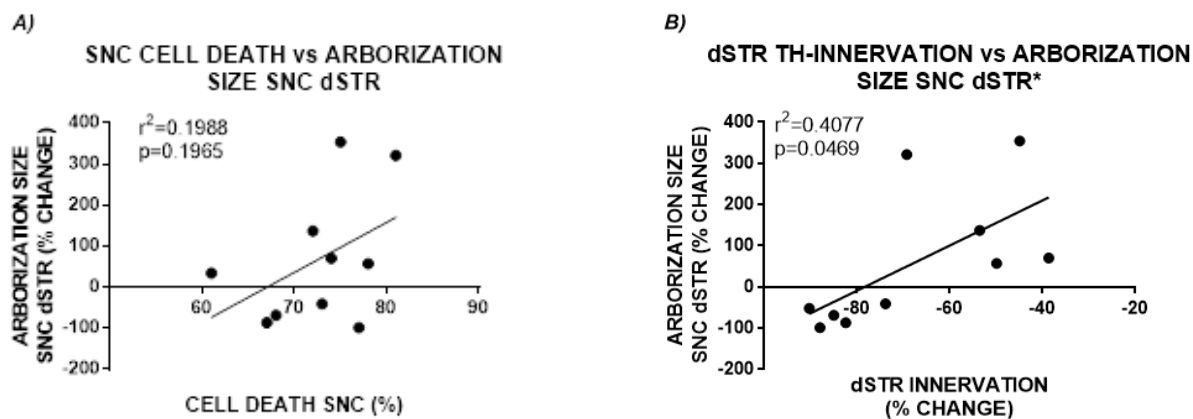
Previous literature (Heuer, 2008) has demonstrated that sprouting of surviving DA neurons can reinnervate and maintain terminal density until $\sim 60\%$ of the neurons are lost. Therefore, following our demonstrated increase in axonal arborization size, we expected this compensation to help reinnervate the partially denervated striatum. We therefore analyzed the relationship between change in arborization size and change in striatal innervation. Level of striatal innervation was calculated using the same confocal images as described above, utilizing

instead the TH signal as opposed to the GFP signal. These images were thresholded to eliminate noise and used to calculate the percent surface area covered by the TH signal. For the following discussion, based on the method of our calculations, striatal innervation will be considered as percent change in TH surface area for the sake of simplicity and clarity.

To determine whether the level of dopaminergic cell loss in the SNC could predict the change in arborization size of neurons projecting the dSTR (*Figure 20A*), a linear regression analysis was performed (n=10). This analysis revealed that whereas there was a tendency, it was not significant ($R^2=0.1988$, $p=0.196$). This is consistent with the literature, whereby level of striatal innervation has shown to be a much better predictor of axonal sprouting than level of cell death (Blanchard et al., 1996; Finkelstein et al., 2000; Lee et al., 2008).

A subsequent linear regression analysis revealed that change in striatal TH-innervation could significantly predict the change in arborization size of targeted SNC-infected neurons projecting to the dSTR (n=10, $R^2=0.4077$, $p=0.0469$). As expected, when striatal TH-innervation was reduced by more than 75%, no increase in arborization size was detectable. In fact, these subjects (5/10) actually demonstrated a reduction in the size of their axonal arbor (*Figure 20B*). This is consistent with the literature suggesting that compensatory mechanisms start to fail with more severe lesions (Blanchard et al., 1996; Finkelstein et al., 2000; Lee et al., 2008).

Figure 20: Linear regression analysis of SNC cell death and dSTR TH-innervation in relation to change in axonal arborization size of SNC DA neurons projecting to the dSTR

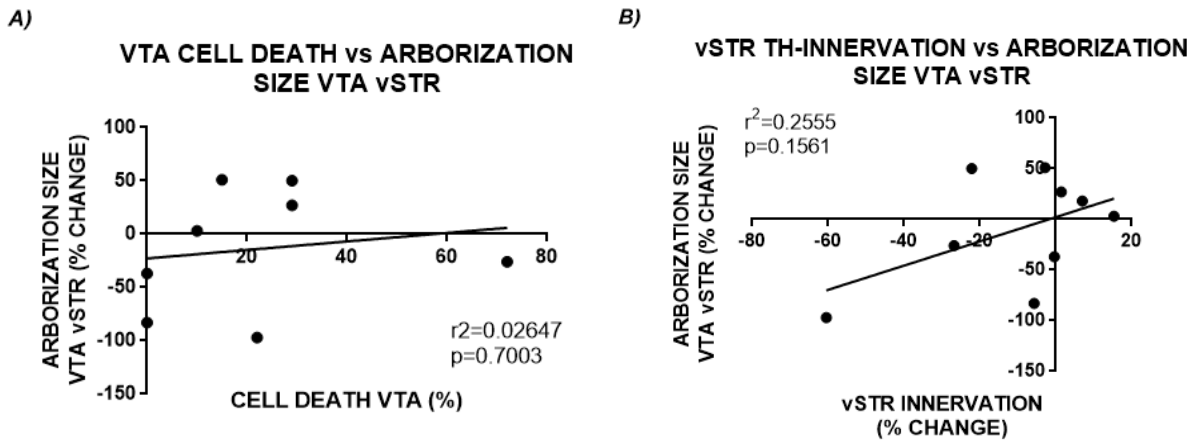


(Figure 20) Linear regression analysis of SNC cell death and dSTR TH-innervation in relation to change in axonal arborization size of SNC DA neurons projecting to the dSTR. (A) A tendency was observed between the level of cell loss in the SNC and the change in arborization size of SNC DA neurons projecting to the dSTR (n=6f; 4m), however, statistical analysis revealed this relationship to not be significant ($R^2=0.1988$, $p=0.1965$). (B) The relationship between the change in dSTR TH-innervation and change in axonal arborization size of SNC DA neurons projecting to the dSTR (n=6f; 4m) was found to be significant in this analysis ($R^2=0.4077$, $p=0.0469$).

To determine whether the level of VTA cell death and vSTR TH-innervation could predict the arborization size of VTA DA neurons projecting to the vSTR, a linear regression analysis was again performed on these variables. The percentage of cell death of VTA DA neurons could not significantly predict the change in arborization size of VTA-targeted DA neurons to the vSTR ($R^2=0.0264$, $p=0.7003$). Interestingly, 2/8 of these subjects had no cell death in the VTA yet demonstrated a 37-83% reduction in arborization size of VTA vSTR DA neurons (*Figure 21A*). This could potentially be explained by partial cell death as opposed to complete cell death in the VTA. In other words, it is possible that at the time the subject was sacrificed these DA neurons were in fact dying, and therefore shrinking, but still contained enough TH phenotype to be counted by stereology.

The change in vSTR TH-innervation also could not predict the change in arborization size of VTA DA neurons projecting to the vSTR ($R^2=0.2555$, $p=0.1651$). This is not surprising given that vSTR TH-innervation was reduced up to a maximum of 30% (aside from one outlier at 60%), which corresponds with the maximum lesion size of ~30% of VTA DA neurons (again aside from one outlier). This outlier of 60% denervation is the result of a 72% lesion to VTA DA neurons in one subject. Increases in arborization size of VTA DA neurons projecting to the vSTR were observed in 4/9 subjects and corresponded to increases in vSTR innervation (*Figure 21B*).

Figure 21: Linear regression analysis of VTA cell death and vSTR TH-innervation vs change in arborization size of VTA-targeted DA neurons projecting to the vSTR



(Figure 21) VTA cell death and vSTR TH-innervation vs change in arborization size of VTA-targeted DA neurons projecting to the vSTR. (A) Linear regression analysis of VTA cell death in relation to change in arborization size of VTA DA neurons projecting to the vSTR ($n=4f$; 4m) was not significant ($R^2=0.0264$, $p=0.7003$). (B) The same statistical analysis of vSTR TH-innervation and change in arborization size of VTA DA neurons projecting to the vSTR ($n=5f$; 4m) was also not significant ($R^2=0.2555$, $p=0.1561$).

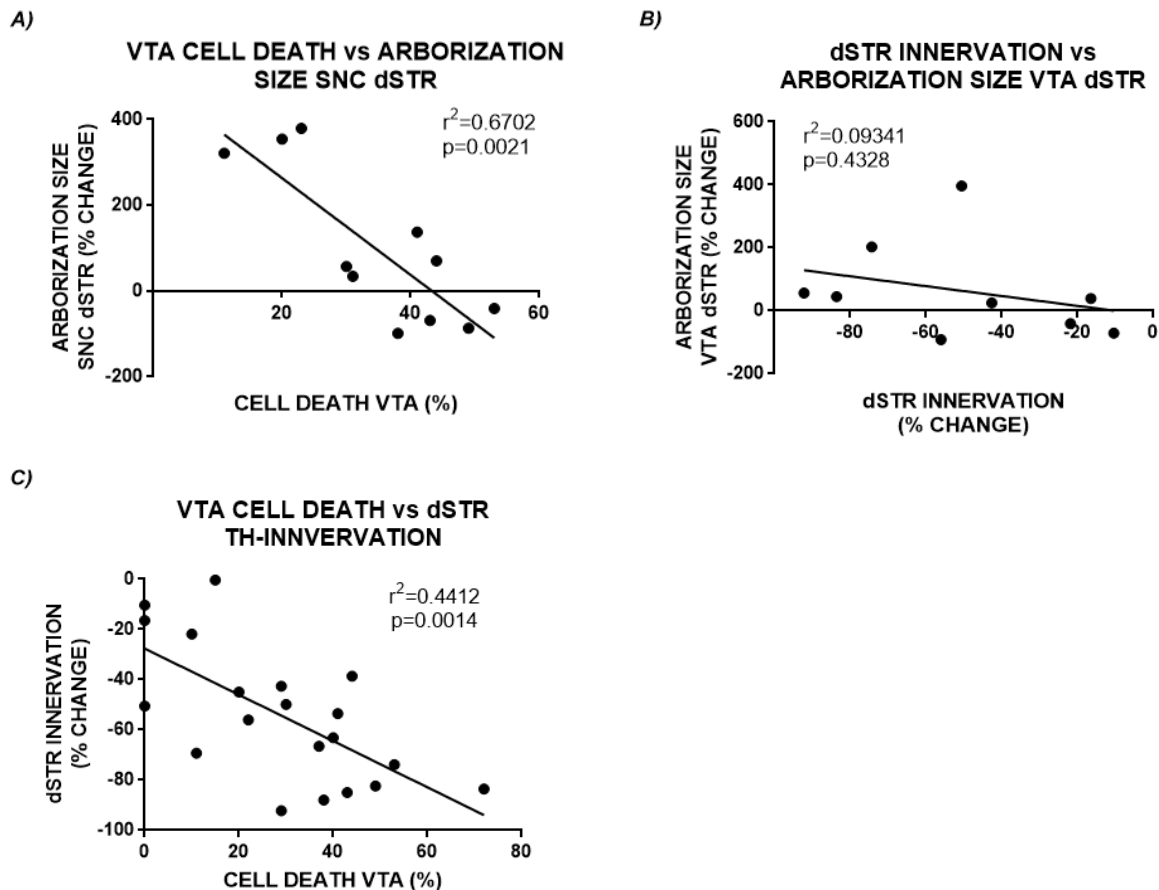
Given that studies have shown that cell death in the VTA can affect compensatory sprouting of SNC DA neurons (Ho et al., 1998; Schneidman et al., 2003; Arkadir, 2014), we also performed a linear regression analysis to determine if the percentage of cell death in the VTA could predict the change in axonal arborization of SNC-infected neurons projecting to the dSTR (Figure 22A). Interestingly, a strong significant negative relationship was found here ($R^2=0.6702$, $p=0.0021$). The higher the cell death in the VTA, the smaller the change in arborization size of SNC-infected neurons projecting to the dSTR. As long as dopaminergic cell death in the VTA remained below 30%, increases in axonal arborization size of SNC neurons could be observed as high as 379% (3/10 subjects). From 30-50% cell death in the VTA however, the effects of SNC arborization became more variable. In 3/7 cases no compensatory sprouting was observed in this range, and the remaining 4 subjects demonstrated lower levels of compensatory sprouting (50-137% increase) in SNC DA neurons.

We next wanted to determine whether the level of dSTR TH-innervation could predict change in arborization size of targeted VTA-infected neurons projecting to the dSTR (*Figure 22B*). In this case, whereas a trend was observed, the relationship between these two factors was not statistically significant ($R^2=0.09341$, $p=0.4328$). This is in contrast to the SNC, where level of dSTR TH-innervation was a better predictor of the arborization size of SNC DA neurons projecting to the dSTR (*Figure 20B*).

Consistent with this, we also found that VTA dopaminergic cell death was a strong predictor of the level of TH-innervation in the dSTR ($n=20$, $R^2=0.4412$, $p=0.0014$). In this case, the greater the amount of cell death of VTA DA neurons, the greater the level of TH-denervation in the dSTR. So oddly, in this case, the level of cell death of VTA DA neurons is a better predictor of dSTR TH-innervation than the level of SNC cell death (*Figure 23A*) ($R^2=0.395$, $p=0.0017$).

These three results taken together provide a relatively strong argument for the influence that VTA DA neurons appear to have on compensating SNC DA neurons and the dSTR. Given the consistency of these results, it seems unlikely that a small amount of potential cross-contamination between SNC and VTA-infected neurons could account for such significant and consistent changes in: VTA cell death on the arborization size of SNC DA neurons projecting to the dSTR (*Figure 22A*) and on the level of VTA cell death predicting the level dSTR innervation (*Figure 22C*).

Figure 22: Linear regression analysis of lesion size and TH-innervation of dSTR in relation to axonal arborization size of VTA DA neurons projecting to the dSTR



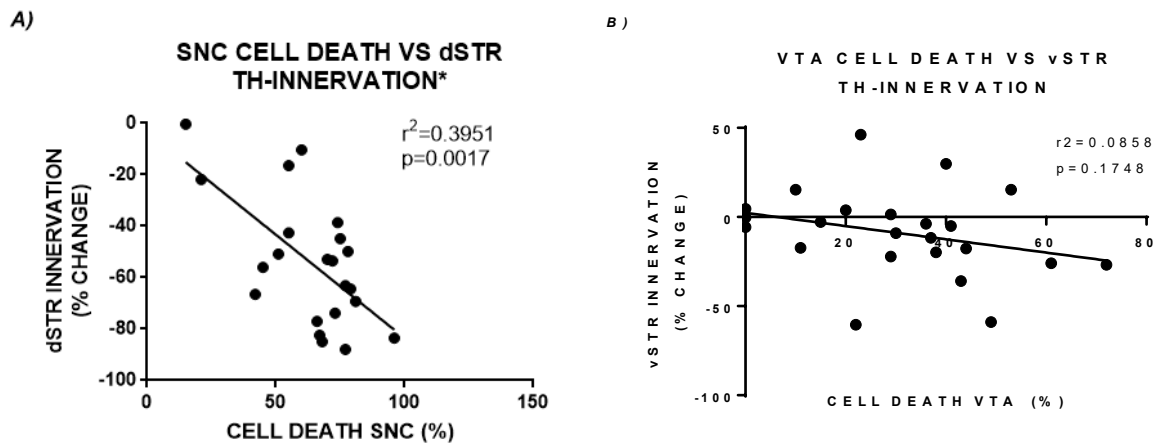
(Figure 22) Lesion size and dSTR TH-innervation in relation to change in axonal arborization size of VTA DA neurons projecting to the dSTR. (A) Linear regression analysis revealed a strong significant negative relationship between level of dopaminergic cell death in the VTA (n=5f; 6m) and change in axonal arborization size of SNC DA neurons projecting to the dSTR ($R^2=0.6702$, $p=0.0021$). (B) A weak negative non-significant relationship was found between change in TH-innervation of the dSTR (n=5f; 4m) and change in axonal arborization size of VTA DA neurons projecting to the dSTR ($R^2=0.09341$, $p=0.4328$). (C) A strong significant relationship was observed between level of VTA dopaminergic cell death (n=11f; 9m) and change in dSTR innervation ($R^2=0.4412$, $p=0.0014$)

Looking at more direct effects, it has been suggested in the literature that the level of cell death in the SNC and VTA following partial lesion can predict the level of striatal denervation in the dSTR and vSTR respectively (Stanic et al., 2003; Porritt et al., 2005). This

theory seems relatively intuitive and was expected. Therefore, we performed a linear regression analysis on these two factors. Our data indicated a significant relationship between TH+ cell death in the SNC (*Figure 23A*) and the change in TH innervation in the dSTR ($R^2=0.395$, $p=0.0017$). The lesion size in this group ranges from 15-96% dopaminergic cell death in the SNC with a change in dSTR TH-innervation ranging from 0-83% ($n=22$). Based on this, our data does appear consistent with literature on predictability.

A linear regression analysis was also performed to examine the relationship between TH+ cell death in the VTA (*Figure 23B*) and the corresponding denervation in the vSTR. This correlation was not significant however ($R^2=0.0858$, $p=0.1748$). Given that up until ~45% cell death in the VTA, the majority of subjects demonstrated less than 20% vSTR TH-denervation, this could be an indication of some form of compensating mechanism occurring in vSTR following VTA cell death. Even a lesion as high as 72% only resulted in a drop in TH-innervation of 26%. This would be an interesting point to further explore in future experiments.

Figure 23: Linear regression analysis of cell death in the SNC and VTA in relation to TH-innervation in the dSTR and vSTR respectively



(Figure 23) SNC and VTA cell death in relation to TH-innervation of dSTR and vSTR. (A) Linear regression analysis was performed examining the relationship between level of dopaminergic cell death in the SNC and change in dSTR TH-innervation ($n=13f$; 9m). This analysis found a strong negative significant relationship between these two variables ($R^2=0.395$, $p=0.0017$). (B) Linear regression analysis between level of dopaminergic cell death in the VTA

and change in vSTR TH-innervation (n=13f; 9m) however, was not significant ($R^2=0.0858$, $p=0.1748$).

Overall, this experiment was able to establish that partial unilateral lesion to the SNC of neonatal mice induced a 2.1-increase in the arborization size of SNC DA neurons projecting to the dSTR, as compared to the unlesioned hemisphere. No significant change in VTA DA neurons projecting to the vSTR was observed, however a trend was present in VTA DA neurons projecting to the dSTR. Our linear regression analysis also corroborated the current literature whereby level of striatal innervation has been shown to be a better predictor of axonal sprouting than level of cell death. In this case, the level of dSTR striatal innervation was able to significantly predict the level of axonal sprouting of SNC DA neurons projecting to the dSTR, whereas only a tendency was observed with level of cell death. In the case of VTA DA neurons projecting to the vSTR, neither level of cell death or TH-innervation was able to significantly predict compensatory axonal sprouting.

Interestingly, this experiment also demonstrated a significant relationship between dopaminergic cell death in the VTA and change in arborization size of SNC DA neurons projecting to the dSTR. In addition, VTA dopaminergic cell death was also found to be a strong predictor of the level of TH-innervation in the dSTR. Overall, these results suggest that compensatory mechanisms may also be activated in VTA dopaminergic neurons, and that there may be a much more complex and intertwined relationship between SNC and VTA DA neurons, and their projections, than was previously thought. Consistent with this theory, the level of cell death of VTA DA neurons was not able to predict level of TH-denervation in the vSTR. Our data suggests that VTA DA neurons may also be able to compensate in their projections to the vSTR.

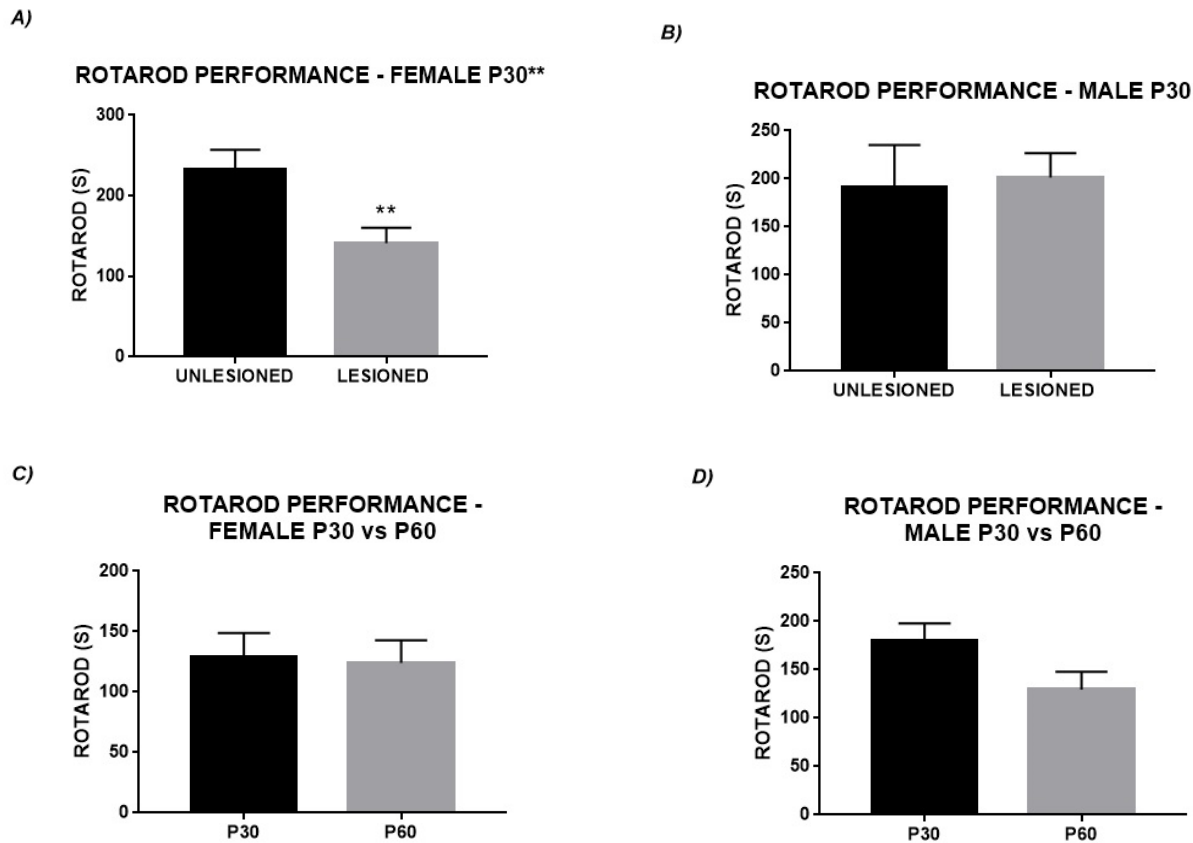
ANALYSIS OF MOTOR FUNCTION IN PARTIALLY LESIONED AND UNLESIONED MICE

ROTAROD PERFORMANCE

There is considerably conclusive literature with regards to detecting parkinsonian behaviour in mice with severe or near-complete unilateral lesions. The literature however, is somewhat inconclusive and contradictory regarding the detection of these behaviors in mice with partial unilateral lesions (Hefti, Melamed & Wurtman, 1980; Carman, Gage & Shults, 1991; Barnéoud et al., 2000). The literature is also somewhat scarce regarding behavioural recovery following partial unilateral lesions. We therefore decided to include several standard behavioural tests to evaluate parkinsonian symptoms (if any) in partially lesioned mice and any potential behavioural recovery that could result from compensation. The following results compare unlesioned to lesioned animals, at P30 and P60 timepoints, in separated female and male subjects. Aside from actimetry measures, the primary behavioural tests that produced significant results were the rotarod, preferential forepaw use, apomorphine-induced rotations and the grip test.

Rotarod performance was used as a test of motor coordination because reduced motor coordination has been reported as a common parkinsonian symptom. Rotarod performance, measured as the amount of time taken to fall from the rotating rod, was expectedly significantly better in unlesioned animals than lesioned. The average rotarod duration was 233s in the unlesioned female P30 group (*Figure 24A*) as compared to 141s in the lesioned group ($p=0.0063$). There was no significant difference found in the P30 male group (*Figure 24B*) between unlesioned and lesioned subjects ($p=0.7343$). In testing to see if compensation could be detected in behavioural recovery following partial lesion, a subset of subjects were tested at two different time points: P30 & P60. No significant difference was found between lesioned female P30 and P60 (*Figure 24C*) subjects. There was also no significant difference found between lesioned male P30 (*Figure 24D*) and P60 subjects ($p=0.083$).

Figure 24: Rotarod performance in unlesioned and partially lesioned, female and male mice at P30 & P60

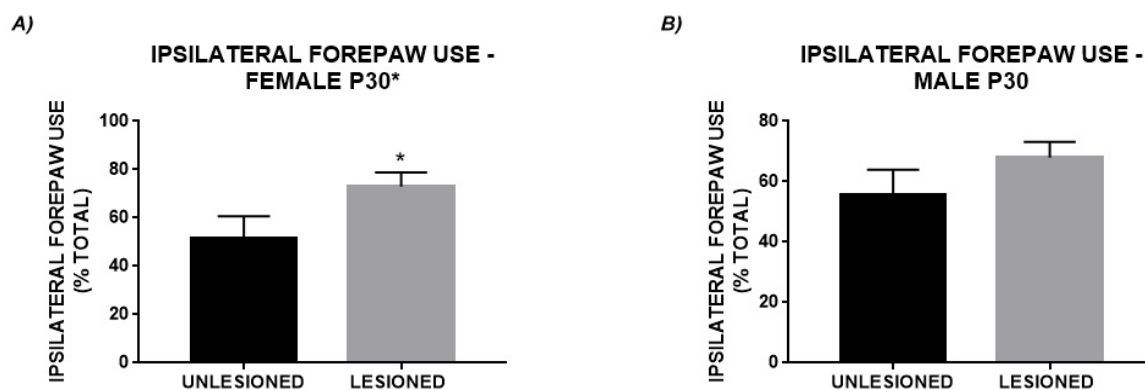


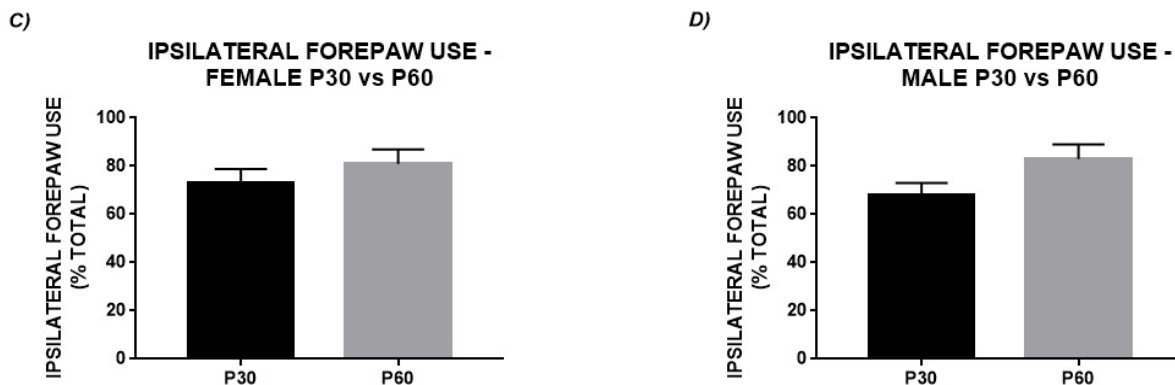
(Figure 24) The rotarod test was used to measure motor coordination, which, in parkinsonian subjects, is reduced. An unpaired t-test analysis was performed on rotarod performance of unlesioned and lesioned female P30 mice ($p=0.0063$). Unlesioned P30 females ($n=18$) had an average of 233s duration on the rotarod, whereas lesioned ($n=16$) females lasted an average of 141s (A). No significant difference was detected between unlesioned ($n=13$) and lesioned ($n=28$) P30 males. Unlesioned males in this group (B) had an average duration of 198s and 185s in the lesioned group ($p=0.7343$). No significant differences were also found in comparing P30 and P60 age groups (C & D). Lesioned female P30 mice ($n=16$) had an average duration of 141s on the rotarod, as compared to 145s in the P60 group ($n=11$, $p=0.8866$). Lesioned males had a slight drop in performance at the P60 time point. Male P30 mice lasted an average duration of 179s on the rotarod, as compared to 129s in the P60 group ($p=0.083$).

PREFERENTIAL FOREPAW USE

Subjects with unilateral lesions tend to exhibit preferential forepaw use of the paw ipsilateral to the lesion, which is considered a parkinsonian symptom. Preferential forepaw use was measured during the cylinder test and calculated as the number of weight-bearing ipsilateral forepaw wall touches divided by the combined ipsilateral and contralateral forepaw wall touches in a 10-minute period. Rearing with weight-bearing wall touches by both paws was not factored into this calculation. Statistical analysis was not performed on results of forepaw use on mice that received apomorphine because, whereas apomorphine administration had a large effect on rotation and drastically increased the number of rotations for a 10-minute period, it nearly eliminated any rearing behavior from which to measure forepaw use. A significant difference was found in preferential ipsilateral forepaw use (*Figure 25A*) between unlesioned and lesioned female P30 mice with no apomorphine ($p=0.0480$), with a 21% increase recorded in ipsilateral forepaw use in lesioned subjects. In contrast, a 12% increase in ipsilateral forepaw use found between unlesioned and lesioned male P30 (*Figure 25B*) mice ($p=0.2241$). When comparing the two time points, the 9% increase in preferential forepaw use between lesioned female P30 & P60 subjects (*Figure 25C*) was not found to be significant ($p=0.3518$). The 16% increase observed between lesioned male P30 & P60 mice (*Figure 25D*) was also not significant ($p=0.0624$).

Figure 25: Preferential forepaw use in unlesioned and partially lesioned female and male mice at P30 & P60





(Figure 25) Preferential forepaw use in unlesioned and partially lesioned mice. Unpaired t-test statistical analysis revealed a significant difference between unlesioned and lesioned female P30 mice (*A*). As expected, unlesioned ($n=14$) female P30 mice used their ipsilateral paw 51% of the time on average, whereas lesioned ($n=21$) females used it an average of 72% of the time ($p=0.0480$). There was a tendency observed in unlesioned and lesioned male P30 mice (*B*), however the difference was not statistically significant ($p=0.2241$). Unlesioned males ($n=8$) used their paw ipsilateral to the lesion 55% of the time, whereas lesioned ($n=21$) subjects exhibited preferential use 67% of the time. Although tendencies were also observed, no significant differences were found when comparing different age groups (*C* & *D*). As stated, the lesioned female P30 group exhibited preferential use 72% of the time, as compared to 81% in the P60 ($n=24$) group ($p=0.3418$). Preferential forepaw use also increased in male P60 subjects ($n=19$) with ipsilateral preferential use 83% of the time ($p=0.0624$).

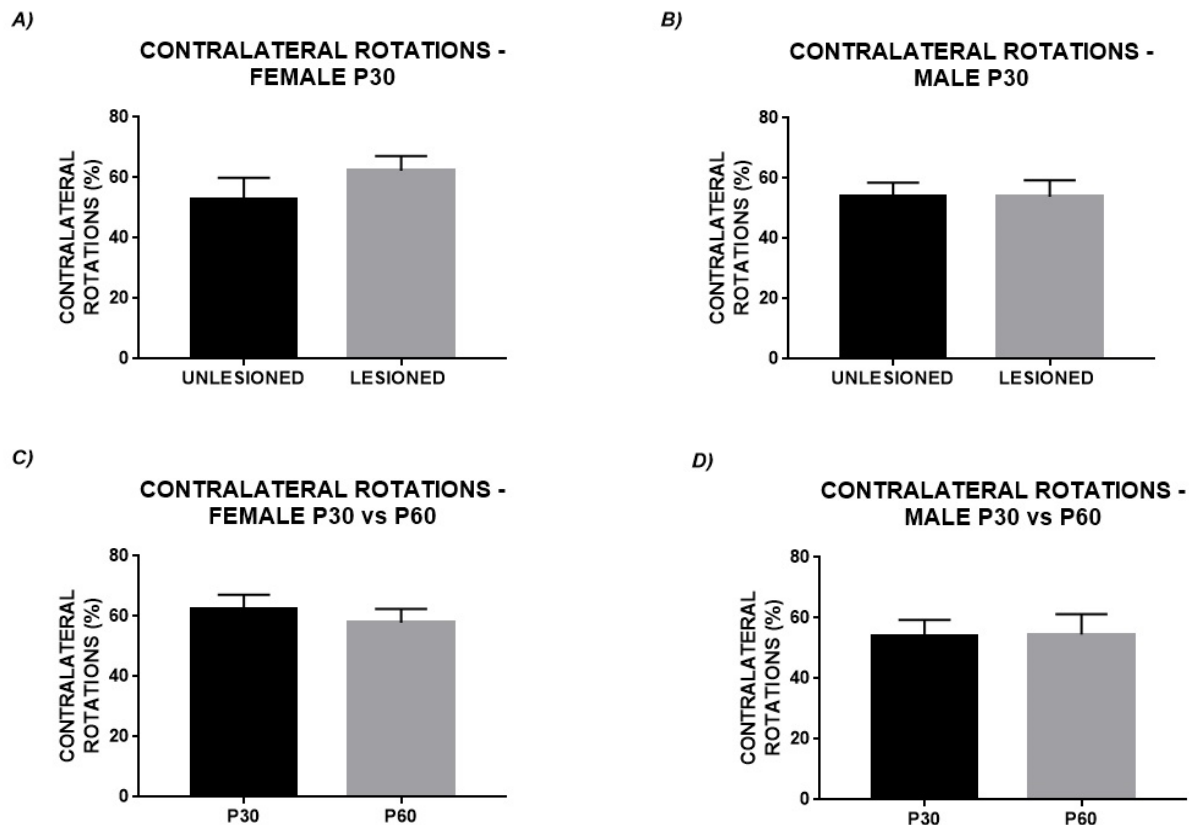
ASYMMETRICAL ROTATION BEHAVIOUR

With severe unilateral lesions, spontaneous rotation behaviour is often observed. This test has also been considered by some studies as reliable in predicting the level of cell death following unilateral partial lesion, however this was not supported by our results (Carman, Gage & Shults, 1991; Formaguera et al., 1994; Barnéoud et al., 2000). It should be noted however, this analysis includes a wide range of lesion sizes combined, so these results may have been diluted. As a parkinsonian symptom, unilateral lesions can induce spontaneous rotation in the direction contralateral to the lesion.

A 10% increase in contralateral rotations was observed in lesioned female P30 mice as compared to unlesioned subjects (*Figure 26A*), however this increase was not significant ($p=0.2672$). No change in rotational behaviour was observed between unlesioned and lesioned

male (*Figure 26B*) P30 mice ($p=0.9946$). Only a 4% decrease in contralateral rotation was found when comparing female P30 & P60 lesioned (*Figure 26C*) subjects ($p=0.5147$), and no difference was found in the male (*Figure 26D*) subjects ($p=0.9343$). No significant differences were found by the t-test analysis between any of these groups.

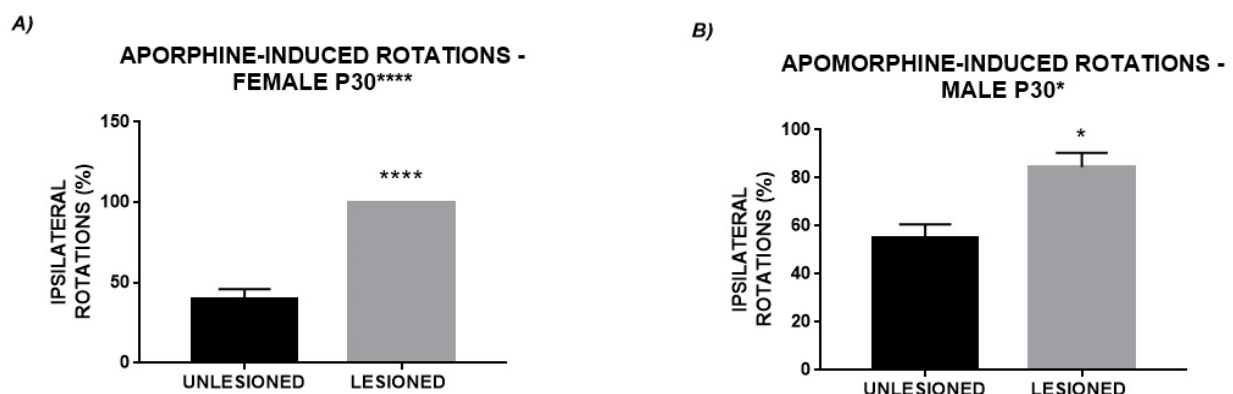
Figure 26: Spontaneous contralateral rotations in unlesioned and lesioned, female and male mice at P30 & P60 – No apomorphine

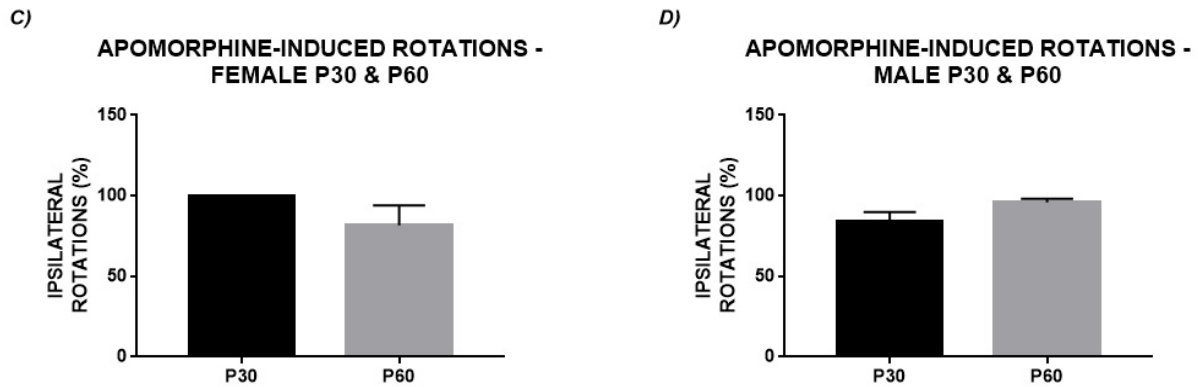


(Figure 26) Spontaneous contralateral rotations in partially lesioned mice. Unlesioned female P30 ($n=14$) subjects did not exhibit asymmetrical rotations (*A*), with contralateral rotations occurring 52% of the total as compared to 62% in the lesioned female group ($n=21$, $p=0.2672$). Both unlesioned ($n=8$) and lesioned ($n=21$) male P30 subjects (*B*) rotated in the contralateral direction 53% of the time ($p=0.9946$). Contralateral rotations in lesioned ($n=24$) female P60 subjects (*C*) occurred 58% of the time, not significantly different from the lesioned P30 group ($p=0.5147$). These rotations occurred 54% of the time in both unlesioned and lesioned males (*D*) of both age groups ($p=0.9343$). None of the differences was statistically significant between groups for this measure.

Apomorphine has been reported to be effective at inducing rotations, in some studies, in subjects with severe lesions. Typically, however, this is reported with lesions larger than 90% and in the direction ipsilateral to the lesion (Hefti, Melamed & Wurtman, 1980; Carman, Gage & Shults, 1991; Barnéoud et al., 2000). We tested this on a small subset of mice, and our results do not support this literature. Apomorphine tended to induce stereotypical-type rotations in the direction ipsilateral to the partial lesion nearly 100% of the time. The unpaired t-test statistical analysis revealed a significant relationship between unlesioned and lesioned female P30 mice (*Figure 27A*), with an increased ipsilateral apomorphine-induced rotation of 60% in the lesioned subjects ($p < 0.0001$). This difference was also statistically significant in unlesioned and lesioned male P30 mice (*Figure 27B*) administered apomorphine with a 29% increase in lesioned subjects ($p = 0.0334$). The statistical significance however, disappears when comparing lesioned female P30 & P60 mice on apomorphine (*Figure 27C*), with a 19% drop in ipsilateral rotation in the P60 group ($p = 0.2673$). This lack of significance also applied to lesioned male P30 & P60 mice (*Figure 27D*) with a 12% increase observed in P60 subjects ($p = 0.1718$). As stated, these results are inconsistent with the current literature, however, the current literature is largely based on DA depletion in adult mice, not neonates. The differential adaptation of our neonates could potentially account for this discrepancy. These results will be further explored in the discussion.

Figure 27: Apomorphine-induced ipsilateral rotations in unlesioned and lesioned, female and male mice at P30 & P60





(Figure 27) Apomorphine-induced ipsilateral rotations in partially lesioned mice. Apomorphine-induced rotations in the direction ipsilateral to the lesion occurred 40% of the time in unlesioned ($n=4$) female P30 subjects (A), as compared to 100% of the time in lesioned ($n=4$) females ($p<0.0001$). These rotations occurred 55% of the time in unlesioned ($n=4$) male P30 subjects (B) and yet consisted of 84% of the total rotations in lesioned males ($n=18$, $p=0.0334$). When comparing two time points, total ipsilateral rotations dropped to 81% in the female P60 ($n=6$, $p=0.2673$) group (C) and increased to 95% in the male P60 (D) group ($n=8$, $p=0.1718$). Neither of the differences between age groups was statistically significant.

GRIP STRENGTH TEST

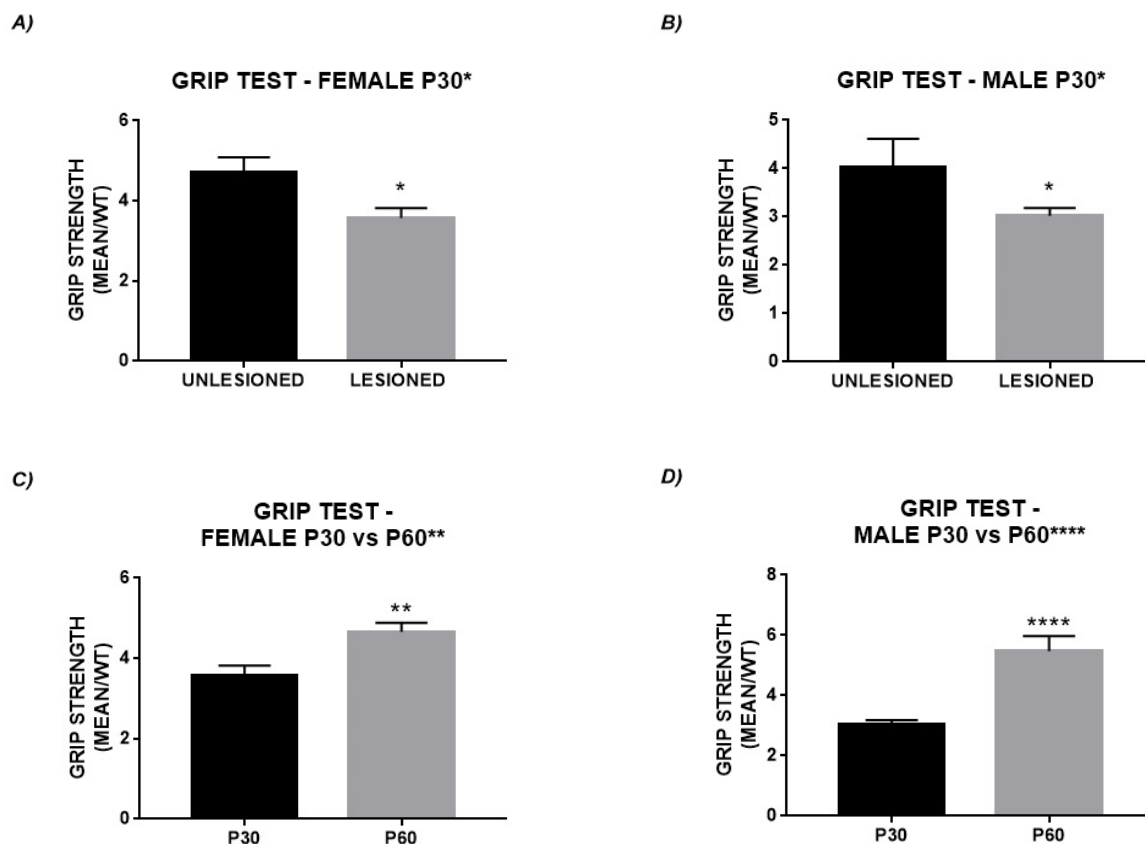
The grip strength test has been used to measure parkinsonian symptoms induced by DA depletion, which is indicated by a marked weakness in grip strength. This was measured in three consecutive tests, which were averaged and divided by the subjects' weight. This was a method of standardizing the measure by weight, allowing distinctions to be drawn between males and females, as well as different age points. Our results demonstrate that this test was able to detect changes in grip strength following partial unilateral lesions.

An unpaired t-test revealed a statistically significant difference between unlesioned and lesioned female P30 subjects ($p=0.0220$) with a drop in the grip strength ratio of 1.2 (Figure 28A). The grip strength ratio also dropped by 1.0 between unlesioned and lesioned male P30 (Figure 28B) subjects ($p=0.0297$). Significant differences were also found between lesioned P30 & P60 mice of both sexes (Figure 28 C&D), with a 1.1 and 2.4-point increase observed in grip strength ratio, respectively (female $p=0.0051$; male $p<0.0001$). It should be noted that, in this case, both the strength and body weight of the subjects increased from P30 to P60, however, proportionally speaking, it seems there is actually an improvement in grip strength over time.

To test whether this could be related to compensatory recovery or just normal aging, a comparison was performed between unlesioned female P30 & P60 (*Figure 29A*) mice ($p=0.0380$), as well as unlesioned male P30 & P60 (*Figure 29B*) mice ($p=0.9979$). As seen in *Figure 29*, this difference was significant for females, exhibiting a 1.0 drop from P30 to P60 age groups, but not for males, which exhibited no difference between groups.

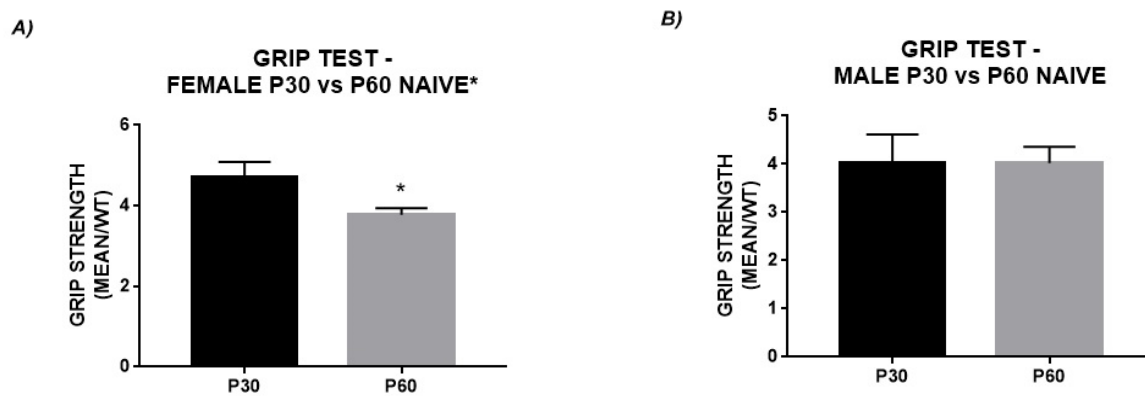
Interestingly, when comparing unlesioned P60 females to lesioned P60 females, the lesioned group outperformed the unlesioned group with a calculated grip strength of 4.6 and 3.5 respectively. This opposite trend in unlesioned females between age groups (the drop in grip strength as age increases as opposed to the increase in grip strength observed in lesioned subjects) indicates that factors other than normal aging are likely contributing to the difference between P30 & P60 age groups. This could be indicative of the previously discussed ‘overcompensation’ effect.

Figure 28: Grip strength test in unlesioned and lesioned mice, female and male, P30 & P60



(Figure 28) Grip strength test in partially lesioned mice. All group comparisons were determined to be statistically significant by the unpaired t-test. Grip strength, as calculated by strength divided by mean body weight, was significantly reduced in lesioned female P30 subjects (*A*). Unlesioned females ($n=8$) had a calculated grip strength ratio of 4.7, whereas lesioned females ($n=23$) were at 3.5 ($p=0.0220$). Unlesioned males ($n=7$) in the P30 group (*B*) had a grip strength ratio of 4.0, as compared to 3.0 in the lesioned ($n=28$) subjects ($p=0.0297$). In comparing age groups, lesioned female P60 ($n=14$) grip strength ratio (*C*) increased to 4.6 ($p=0.0051$), and lesioned male P60 ($n=11$) subjects (*D*) increased to 5.4 ($p<0.0001$).

Figure 29: Grip strength test between females and males, P30 & P60, in unlesioned mice



(Figure 29) Grip strength test in unlesioned females and males of different age groups. The grip strength ratio was 4.7 in unlesioned ($n=8$) female P30 subjects, and 3.7 in unlesioned ($n=8$) P60 female (*A*) subjects ($p=0.0380$). This ratio was stable at 4.0 for both P30 ($n=7$) & P60 ($n=3$) unlesioned (*B*) males ($p=0.9979$).

To summarize, given our hypothesis of compensation in our model of partially lesioned neonatal mice, we included behavioural testing into our experimental design to examine whether behavioural recovery would occur as a result of compensatory mechanisms. This was analyzed by comparing lesioned subjects at two different timepoints, P30 & P60. In addition, as often conducted in experiments involving unilateral lesions, we also used this as an additional comparison of behavioural differences between lesioned and unlesioned mice. Significant

differences were observed between unlesioned and lesioned subjects in the rotarod and preferential forepaw use measures, however no difference was detected between timepoints. Apomorphine-induced rotations have typically been reported to only occur in subjects with severe lesions. In this experiment however, in subjects with varying degrees of partial unilateral lesions, apomorphine induced stereotypical-typed rotations in the direction ipsilateral to the lesion nearly 100% of the time. This could be indicative of differential physiological effects of lesions created in neonates as compared to adults. No significant differences were observed in this measure between timepoints however.

As expected, the grip strength test also revealed a significant reduction in lesioned mice as compared to unlesioned subjects. Unexpectedly however, this test did demonstrate significant differences between P30 and P60 subjects of both sexes, with improved grip strength performance observed in P60 subjects than P30. This was an indication of compensatory recovery. In addition, lesioned P60 females out-performed unlesioned P60 females, indicating there may also be an overcompensation effect occurring.

CELL DEATH AND STRIATAL DENERVATION IN RELATION TO PERFORMANCE ON BEHAVIOUR TASKS

Given that one of the purposes of our behavioural tests in this project was to determine whether compensation could be detected through behavioural recovery, we next sought to analyze whether cell death and level of striatal denervation could predict performance on the various aforementioned behavioural measures. The following analyses were performed in groups of combined sex at age P30. Whereas a number of tendencies did emerge, the only relationship that did demonstrate significance was the level of dSTR innervation in predicting preferential forepaw use (*Figure 30B*). Due to a high level of individual variability in performance between subjects, cell death was not a good predictor of parkinsonian behaviour (*Figure 30 A, C, E, G, I*). Overall, dSTR innervation appeared to be a better predictor of behavioural performance than SNC cell death (*Figure 30B, D, F, H, J*).

As seen in Figure 30A, SNC cell death could not predict level of preferential forepaw use in partially lesioned mice due to high levels of individual variability ($R^2=0.0004383$, $p=0.9322$). Preferential ipsilateral forepaw use did however, increase the more the dSTR became denervated, with many subjects nearing the 100% level after ~60% denervation ($R^2=0.3931$, $p=0.0031$). This is consistent with parkinsonian behaviour (*Figure 30B*).

A tendency was observed in the negative relationship between change in dSTR innervation and ipsilateral rotations without the use of a DA agonist (*Figure 30D*). Unlesioned mice will typically display rotations in approximately equal directions, therefore, consistent with parkinsonian behaviour, these partially lesioned mice displayed an increase in contralateral rotations the as level of SNC cell death became greater ($R^2=0.1201$, $p=0.1461$). The level of striatal denervation in this group ranged from 10-82%, while the number of rotations in the direction ipsilateral to the lesion ranged from 10-88%. Whereas a tendency was evident, there was a large amount of individual variability. Within a range of 63-70% of dSTR denervation, the percentage of ipsilateral rotations ranged from 23-76%. This suggests that in the present experiment, rotation direction was not a great indicator of lesion size. The results do indicate however, that changes in rotation direction can be observed in partially lesioned mice with greater than 40% dSTR denervation in approximately half of the tested mice.

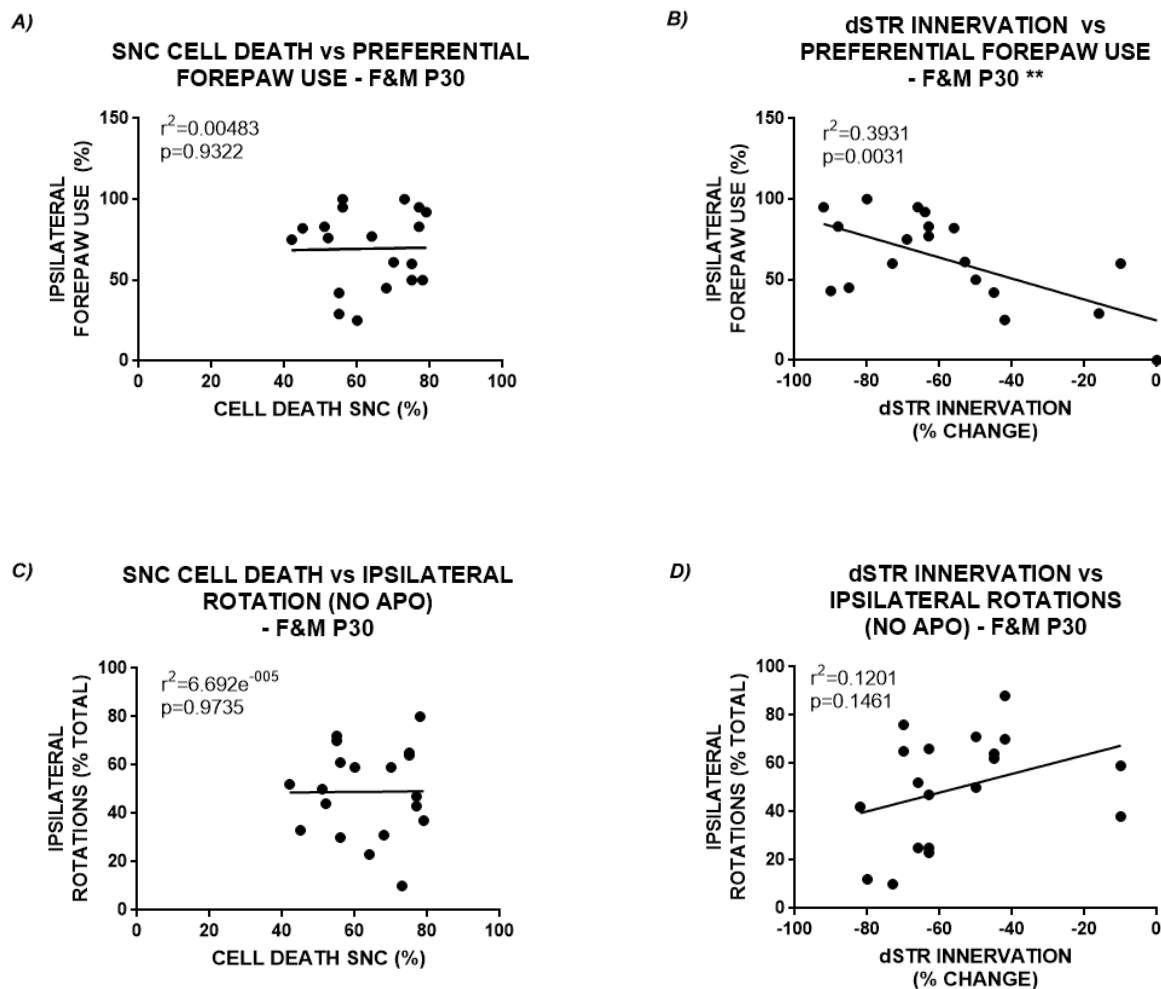
Given that apomorphine administration induced an all-or-nothing result in the rotation of lesioned animals, this measure could be used to predict lesioned animals from unlesioned animals, however not useful for predicting the lesion size (*Figure 30E & 30F*). There was however, a trend observed in the apomorphine-induced rotation behaviour of mice in relation to dSTR denervation (*30F*). As stated, the mechanism of action of apomorphine results in rotations in the direction ipsilateral to the lesion. The trend observed here indicates an increase in the percentage of ipsilateral rotations as the level of dSTR denervation increases ($R^2=0.3844$, $p=0.0749$). In this case, an approximate 20% denervation resulted in ~50% ipsilateral rotation, which would be consistent with unlesioned subjects, but from 45-92% dSTR denervation the majority of subjects rotated in the ipsilateral direction 100% of the time. This again demonstrates that partial lesions can be detected with the use of apomorphine, which is inconsistent with the literature.

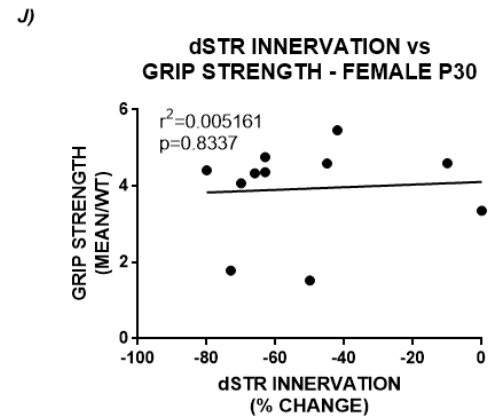
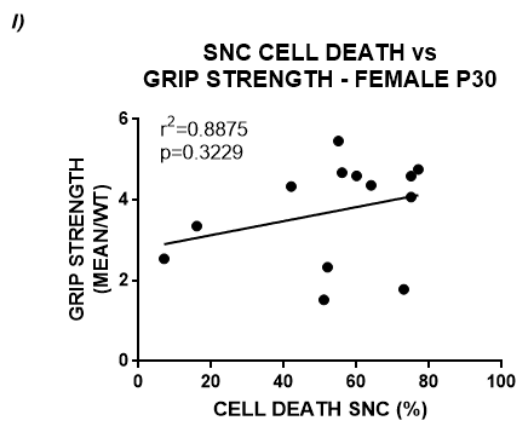
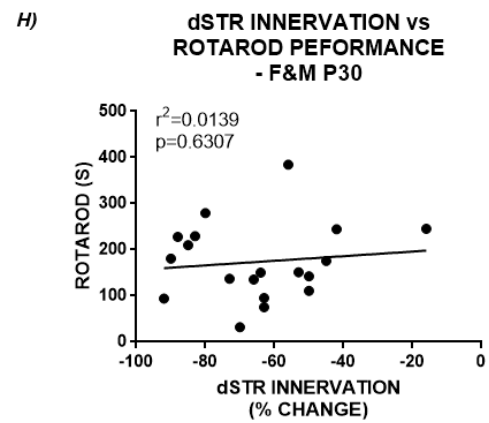
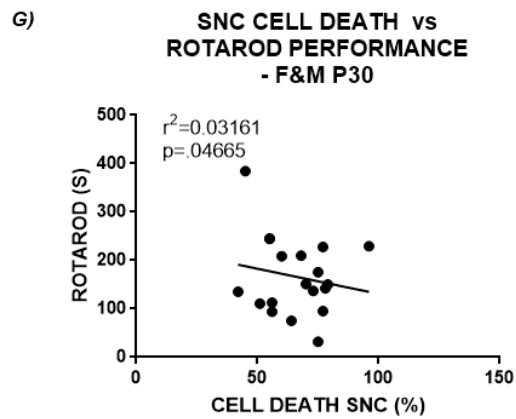
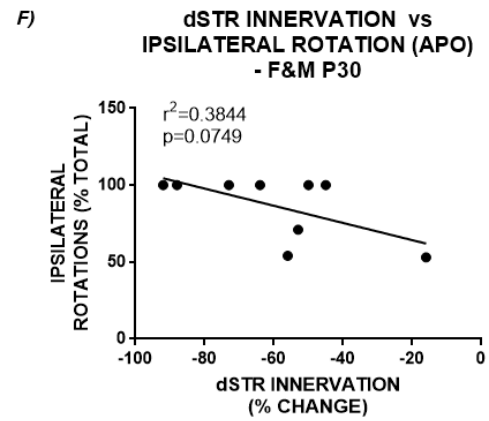
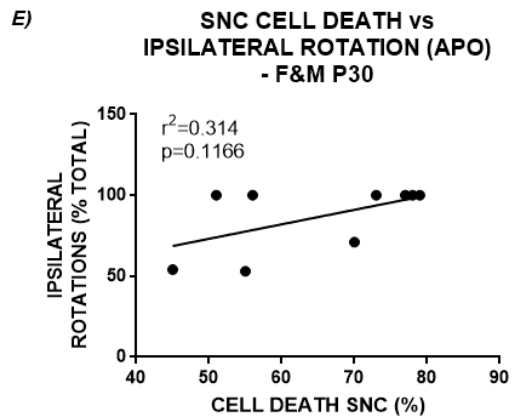
Parkinsonian behaviour predicts that the level of motor coordination should decrease as a result of partial lesions. This behaviour was evaluated using the rotarod test. Again, the level of cell death (*Figure 30G*) was not a significant predictor of rotarod performance ($R^2=0.314$, $p=0.1166$). The level of dSTR denervation also lacked a strong predictive quality due to high individual variability ($R^2=0.3844$, $p=0.0749$), although there was a curious pattern observed in this analysis (*Figure 30H*). Out of 19 subjects ranging from 16-92% dSTR denervation, the performance of the group was split at the 175s mark: approximately half of the subjects lasted less than 175s on the rotarod and the other half lasted longer. The unexpected result however, comes in the analysis of the subjects that lasted longer than 175s. The majority of the subjects (5/6) with striatal denervation greater than 80% lasted longer than 175s. Behavioural recovery is more often expected when striatal denervation is less than 80%, whereas for these subjects, dSTR denervation was between 80-90%. This is surprising given that 5/6 of these mice in the severe lesion range performed better than the majority of mice with 45-75% denervation. It could be suggested that perhaps this improved performance could be partially attributed more to hyperactivity than motor coordination, however the evidence to support this theory at this time is weak.

As stated, another common parkinsonian symptom is muscle weakness. To test this in our partially lesioned mice, we performed a grip strength test. Muscle strength can be influenced by body weight, therefore in order to standardize the results across subjects, the mean grip strength over the three trials was divided by each subjects' body weight. A small amount of variability is typical within a group of subjects of the same sex and age. Due to this, our grip strength analysis kept groups separated based on sex and age. In this case, level of dSTR innervation could not significantly predict grip strength (*Figure 30J & L*) for females ($R^2=0.005161$, $p=0.8337$) or males ($R^2=0.00405$, $p=0.8624$), in fact, similar results were obtained in grip strength no matter the level of denervation. Slight trends were observed in the linear regression analysis of SNC cell death and grip strength (*Figure 30I & K*), for females ($R^2=0.08875$, $p=0.3229$) more so than males ($R^2=0.01028$, $p=0.7952$). This trend however, was in the opposite direction than would have been predicted. In many of these subjects, the larger the lesion in the SNC, the more the grip strength seemed to increase. This would be contradictory to the expectation of increased muscle weakness with increased lesion size. However, the

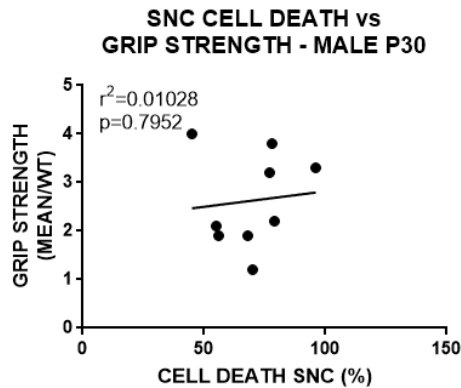
literature has shown increased muscle weakness following unilateral lesion, not necessarily increasing as lesion size increases. It is possible therefore, that these results could actually be indicative of behavioural recovery in larger lesions. Interestingly, this opposite effect is also in line with our previous results on lesion size and rotarod performance. The potential that, in some cases, a larger lesion size can increase compensatory mechanisms to the point behavioural recovery is observed more so in larger lesions than smaller ones is curious. This possibility is further explored in the discussion.

Figure 30: Linear regression analysis of measured parkinsonian behaviors in relation to dopaminergic cell death and dSTR innervation

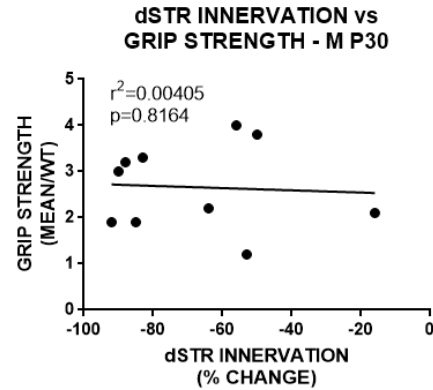




K)



L)



(Figure 30) Parkinsonian behaviours in relation to SNC cell death and dSTR innervation in partially lesioned mice. (A) Level of SNC cell death in relation to preferential forepaw use ($n=19$; $R^2=0.000438$, $p=0.9322$). (B) Change in level of dSTR innervation in relation to preferential forepaw use ($n=20$; $R^2=0.3931$, $p=0.0031$). (C) SNC cell death vs contralateral rotations, no apomorphine ($n=19$; $R^2=6.69e^{-005}$, $p=0.9735$). (D) Change in dSTR innervation vs contralateral rotations, no apomorphine ($n=19$; $R^2=0.1201$, $p=0.1461$). (E) Level of SNC cell death vs apomorphine-induced ipsilateral rotations ($n=9$; $R^2=0.314$, $p=0.1166$). (F) Change in dSTR innervation vs apomorphine-induced ipsilateral rotations ($n=9$; $R^2=0.3844$, $p=0.0749$). (G) Level of SNC cell death vs rotarod performance ($n=19$; $R^2=0.03161$, $p=0.4665$). (H) Change in dSTR innervation vs rotarod performance ($n=19$; $R^2=0.0139$, $p=0.6307$). (I) Level of SNC cell death vs grip strength (mean/weight) in P30 females ($n=13$; $R^2=0.08875$, $p=0.3229$). (J) Change in dSTR innervation vs grip strength in P30 females ($n=11$; $R^2=0.005161$, $p=0.8337$). (K) Level of SNC cell death vs grip strength (mean/weight) in P30 males ($n=9$, $R^2=0.01028$, $p=0.7952$). (L) Change in dSTR innervation vs grip strength in P30 males ($n=10$, $R^2=0.00405$, $p=0.8624$).

In short, neither SNC cell death nor level of TH-denervation was able to significantly predict performance on any of the aforementioned behavioural tests, with the exception of preferential forepaw use and level of TH-denervation in the dSTR. Whereas comparison of the other behaviours and these two factors was not statistically significant, tendencies were observed in the expected direction. The trend observed in grip strength however, was in the opposite direction as expected. In this case, greater increases in grip strength was observed in subjects with greater cell death, suggesting again the occurrence of behavioural recovery, and raising the question of whether, in some cases, compensatory mechanisms induce more obvious behavioural recovery in subjects with larger lesions as opposed to smaller ones. It is important to note that at this point, the link between behavioural symptoms and potential neurobiological

mechanisms of compensation is correlational. Further testing would be required to determine full cause-and-effect relationships with these measures.

LOCOMOTOR MEASURES OF BEHAVIOR

Other parkinsonian symptoms typically include reduced locomotion and increased stereotypic behaviour. To assess this, we conducted actimetry tests on unlesioned and partially lesioned mice. This consisted of 30-minutes spent in an actimeter, with no habituation time. Many measures were automatically recorded, including: horizontal activity count, ambulatory time, ambulatory activity count, stereotypy time, stereotypy episode count, stereotypy activity count, rest time, movement time, margin time and center time. In order to properly understand these measures, the following definitions from the Omnitech manufacturer are relevant:

Total distance: The total distance the subject has traveled. This variable defines the subjects' location as the centroid.

Ambulatory activity count: Number of beam breaks while animal is ambulating. Does not include stereotypy behaviour.

Rest time: The length of time that the subject spends at rest. A resting period is defined as a period of inactivity greater than or equal to 1 second.

Movement time: The length of time that the subject spent in activity. Activity is defined as a period in which ambulation or stereotypy occurred.

Ambulation: Ambulatory activity after a subject's centroid has moved in excess of 5cm from the location of its previous rest episode. Upon crossing this 5cm threshold, the ambulatory activity episode count increases by 1 and the ambulatory time value begins to increment.

Non-ambulatory behaviour time: The total amount of time that the subject repeatedly breaks the same beam or set of beams. This could be potential stereotypic behaviour, however further observation would be required to confirm. A break in repeated behaviour of 1 second or more is required to separate one episode from the next. This typically happens during grooming or head bobbing.

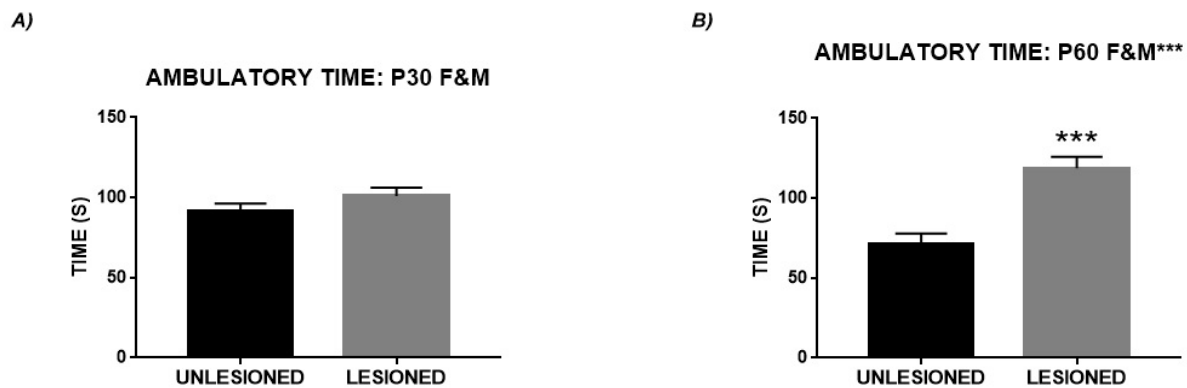
Non-ambulatory behaviour activity count: The number of beam breaks due to repeated non-ambulatory activity.

Margin time: Time spent by the animal in close proximity to the walls of the cage.

Center time: Total time spent in the center portion of the cage.

To determine whether our partially lesioned mice exhibited reduced locomotion as one of their parkinsonian symptoms, unpaired t-tests were performed on these various measures. Only the ambulatory time, ambulatory activity and movement time were statistically significant however. Interestingly, the results obtained were again the opposite of what was expected. Ambulatory time, defined as the amount of time spent in ambulation once the subject moved more than 5cm, was not significantly different between unlesioned (n=25) and lesioned (n=23) P30 mice ($p=0.1792$), with the average time increasing by 9 seconds in the lesioned group (*Figure 31A*). Ambulatory time was statistically significant however between the unlesioned (n=10) and lesioned (n=9) mice in the P60 group (*Figure 31B*), with an average increase of 47s in the lesioned group ($p=0.0006$).

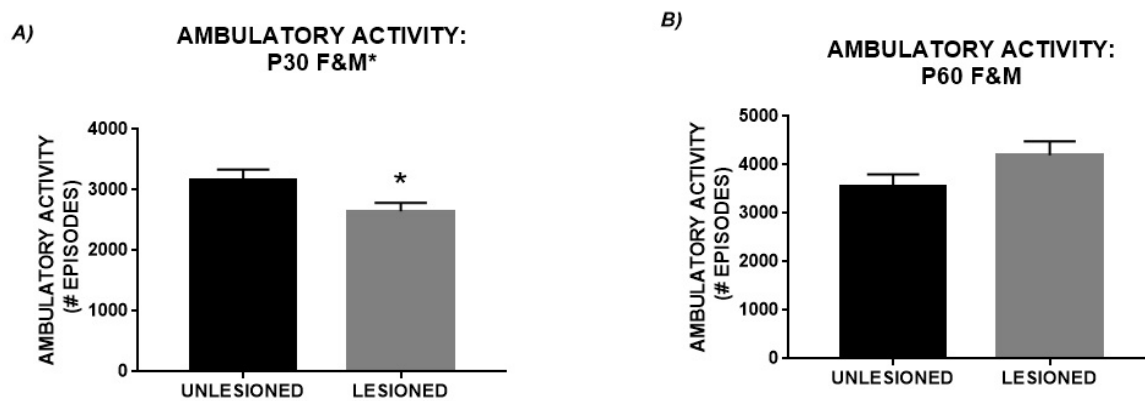
Figure 31: Ambulatory time in P30 & P60 mice



(Figure 31) Ambulatory time in P30 & P60 partially lesioned mice (combined sexes). No significant difference was found in ambulatory time between unlesioned (n=13f; 12m) and lesioned (n=13f; 10m) P30 mice (A). Average ambulatory time in P30 unlesioned subjects was 91s, as compared to 100s in the lesioned group ($p=0.1792$). A significant difference was found in ambulatory time between unlesioned and lesioned P60 subjects (B). Average ambulatory time for unlesioned (n=6f; 4m) P60 subjects was 71s as compared to 118s in the lesioned (n=6f; 3m) group ($p=0.0006$).

Ambulatory activity was also measured in partially lesioned P30 & P60 subjects. This differs from ambulatory time in that it is the number of episodes of ambulatory activity (# of beam breaks) that took place during ambulation. Ambulatory activity was significantly different between unlesioned and lesioned P30 subjects (Figure 32A), with an average decrease of 524 episodes in the lesioned group ($p=0.0205$). The opposite trend was observed in the P60 group (Figure 32B) however, with an average increase of 649 episodes observed in the lesioned group ($p=0.1425$). Taken together with the results of ambulatory time, this would indicate that whereas the ambulatory time was increased in lesioned subjects as compared to unlesioned subjects of both age groups, the overall distance covered was less in the lesioned P30 group. This could indicate that the lesioned subjects of the P30 group were moving with less vigor than the unlesioned subjects. The P60 group however, demonstrated both an increase in ambulatory time and number of episodes, which indicates overall more movement in the lesioned group.

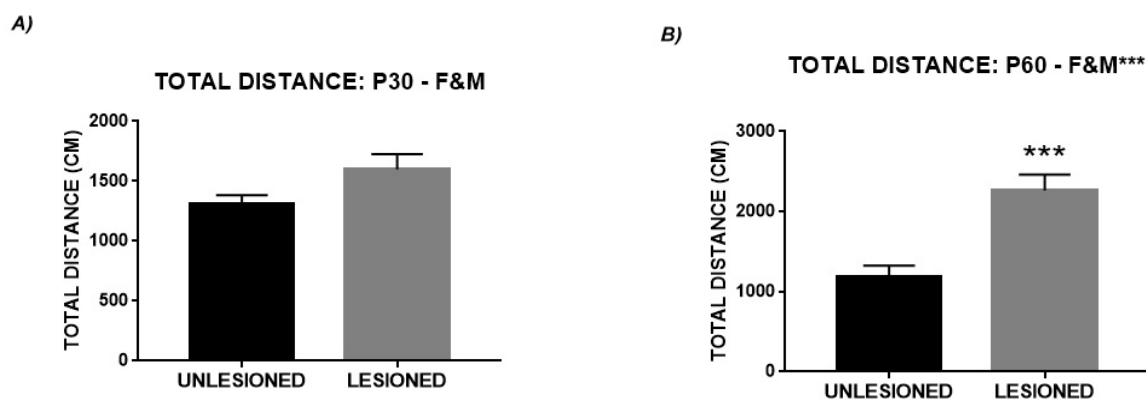
Figure 32: Ambulatory activity in P30 & P60 mice



(Figure 32) Ambulatory activity in partially lesioned P30 & P60 mice. Statistical analysis using an unpaired t-test revealed a significant difference between unlesioned (n=13f; 12m) and lesioned (n=13f; 10m) ambulatory activity in P30 mice (A). Unlesioned subjects exhibited an average of 3169 beam breaks in a 30-minute period, whereas lesioned subjects had reduced ambulatory activity at 2645 episodes ($p=0.0205$). Ambulatory activity in P60 subjects was found to increase in lesioned subjects (B). Unlesioned (n=6f; 4m) mice had an average of 3546 episodes, as compared to lesioned (n=6f; 3m) subjects at 4195 episodes ($p=0.1425$).

The total distance travelled was another measure found to be significant in terms of parkinsonian behaviour. Consistent with ambulation time, these results were also the opposite of what was expected. The difference between unlesioned and lesioned P30 subjects (Figure 33A) was near statistically significant and increased by 288cm in the lesioned group ($p=0.0534$). There was a statistically significant difference in the total distance travelled however between unlesioned and lesioned P60 subjects (Figure 33B), with an increase of 1084cm ($p=0.0008$). The P60 lesioned group on this measure is consistent with the increased ambulatory time and activity variables discussed above. In addition, given that at P30, lesioned subjects appear to move with less vigor, yet at P60 they overall exhibit more movement than unlesioned subjects, this could be an indication not only of behavioural recovery, but potentially an overcompensation effect. Hyperlocomotor activity resulting from compensatory mechanisms induced by DA depletion of neonatal mice could also be a contributing factor. These concepts will be discussed in further detail in the next section.

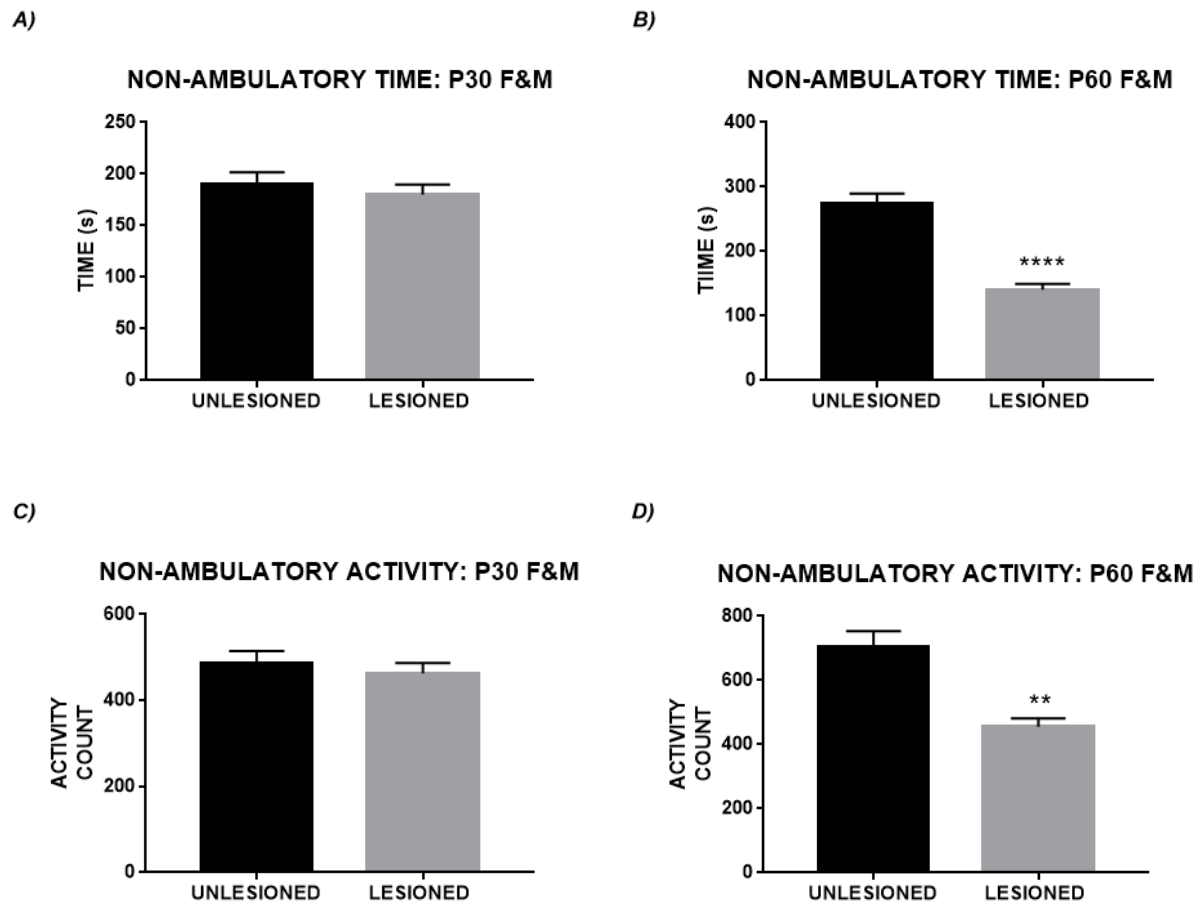
Figure 33: Total distance covered in P30 & P60 mice



(Figure 33) Total distance travelled in partially lesioned P30 & P60 mice. The total distance travelled in unlesioned ($n=13f$; 12m P30 subjects (*A*) was 1311cm, as compared to 1599cm in the lesioned ($n=13f$; 10m) group ($p=0.0534$). The total distance travelled in unlesioned ($n=6f$; 4m) P60 subjects (*B*) was 1180cm, as compared to 2264cm in the lesioned ($n=6f$; 3m) group ($p=0.0008$).

Other significant locomotor activity measures included non-ambulatory behaviour time and non-ambulatory activity count. In this case, non-ambulatory behaviour could be potential stereotypic activity, however further observation would be required to confirm. These measures were all found to be consistent with the previous results of ambulatory activity, ambulatory time and total distance travelled. No significant difference was found between unlesioned and lesioned P30 mice in non-ambulatory behaviour time ($p=0.5118$). There was however, a significant difference in the P60 group, with a reduction of 133s observed in the lesioned group ($p<0.0001$). Consistent with this, there was no significant difference between unlesioned and lesioned P30 mice ($p=0.5291$) in non-ambulatory activity, but there was a significant difference in the P60 group. The non-ambulatory activity count was reduced by 250 in the lesioned group ($p=0.0037$).

Figure 34: Non-ambulatory behaviour time and non-ambulatory activity in P30 & P60 mice



(Figure 34) Non-ambulatory behaviour time and non-ambulatory activity of partially lesioned P30 & P60 mice. Non-ambulatory behaviour time was not significant between unlesioned (n=13f; 12m) and lesioned (n=13f; 10m) P30 groups (A) but was significantly reduced in the lesioned P60 group (B). Average non-ambulatory behaviour time was 190s in the unlesioned group as compared to 180s in the lesioned group ($p=0.5118$). The difference in this time was significant in the P60 group, reduced from 273s in the unlesioned group to 140s in the lesioned group ($p<0.0001$). No change was observed in the non-ambulatory activity count of P30 mice (C), with an activity of 486 in the unlesioned group (n=25), as compared to 462 in the lesioned group ($p=0.5291$). Non-ambulatory activity was significantly reduced in the P60 lesioned group (D) however. The activity count was 705 in the unlesioned (n=6f; 4m) group as compared to 455 in the lesioned (n=6f; 3m) group ($p=0.0037$).

Overall, whereas increased ambulatory time was documented in P30 lesioned subjects, the distance covered was reduced as compared to unlesioned subjects. Interpretation of these results could be that subjects in the lesioned P30 group moved with less vigor than unlesioned subjects. With regards to the P60 lesioned group however, more movement overall was recorded as compared to unlesioned subjects. This was not only unexpected, but an indication of behavioural recovery as well as a potential overcompensation effect. In addition, non-ambulatory behaviour was reduced in lesioned P60 subjects as compared to the unlesioned group, which could also indicate potential behavioural recovery.

To summarize, the purpose of these behavioural tests was to 1) determine if parkinsonian behaviour can be detected in partially lesioned mice and 2) to determine if evidence of compensation could be detected through behavioural recovery following a partial unilateral lesion in mice. As demonstrated, parkinsonian behaviour can be detected to varying degrees with the rotarod test, the measure of preferential forepaw use, apomorphine-induced rotations and the grip strength test. In general, the results from these tests were much better correlated with level of dSTR innervation than level of SNC cell death. Using actimetry, locomotor behaviour was found to be affected following partial lesions, however not in a diminished way as was expected. Particularly in the P60 age group, locomotor behaviour was elevated in comparison to most unlesioned control subjects. This is thought to be explained by either an “overcompensation” effect of the dopaminergic system induced by the partial lesion, or hyperlocomotion induced by compensation in neonatal mice. Therefore, it was demonstrated that some parkinsonian symptoms can be detected through behavioural testing, and evidence of compensation was observed through behavioural recovery following the partial unilateral lesion of our mice.

INCREASED VULNERABILITY OF DA NEURONS

The second hypothesis of this experiment predicted that compensating DA neurons would exhibit increased vulnerability following exposure to a secondary stressor. To test this

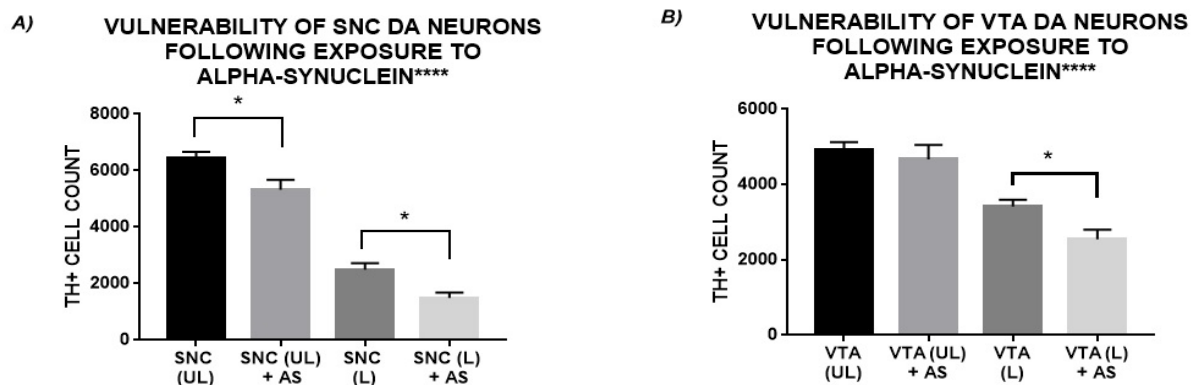
hypothesis, a separate cohort of lesioned mice were bilaterally injected with a virus allowing overexpression of wild-type alpha-synuclein at P60. Brains were then extracted at P135, 75 days post alpha-synuclein viral injection, and then processed for stereological counting. Supporting our hypothesis, a significant increase was found in the relative vulnerability of SNC DA neurons in the lesioned hemisphere as compared to the unlesioned hemisphere following the secondary alpha-synuclein stressor. This increase in vulnerability was calculated by comparing the number of surviving DA neurons in mice that had received only unilateral 6-OHDA injections to mice that had received both unilateral 6-OHDA and bilateral alpha-synuclein injections. The vulnerability of DA neurons was estimated by comparing the number of TH⁺ cells in the untreated unlesioned hemisphere compared to the unlesioned hemisphere that received only alpha-synuclein. The vulnerability of compensating DA neurons was estimated by comparing the number of TH⁺ neurons in the 6-OHDA-only lesioned hemisphere to the number of TH⁺ neurons in the 6-OHDA lesioned hemisphere which also received alpha-synuclein.

As determined by stereological counting, there was a mean of 6445 TH⁺ SNC cells in the unlesioned hemisphere (n=41), compared to a TH⁺ cell count of 5322 in the unlesioned hemisphere following alpha-synuclein exposure (n=16; p=0.0106). This 17% decrease gives us a baseline for the number of normal TH⁺ cells killed following 75 days of alpha-synuclein overexpression. The average TH⁺ cell count in the SNC of the 6-OHDA lesioned hemisphere was 2499 (n=41), compared to an average of 1492 (p=0.0243) in the SNC of 6-OHDA+ alpha-synuclein hemisphere (n=16). This results in a 40% decrease in surviving DA neurons following exposure to the secondary neurotoxin. Overall, when relative changes in cell death are compared, there is an approximate 2.3-fold increase in the vulnerability of compensating DA neurons of the SNC following exposure to secondary stressors (*Figure 34A*).

The same analysis was performed for TH⁺ neurons of the VTA in both 6-OHDA and alpha-synuclein treated mice. Stereological counting determined 4906 TH⁺ cells in the VTA of unlesioned hemispheres, as compared to 4668 TH⁺ cells in the VTA of unlesioned hemispheres following alpha-synuclein exposure (p=0.7801). The 6-OHDA lesioned VTA had an average TH⁺ cell count of 3416, whereas this number dropped to 2543 following alpha-synuclein exposure (p=0.0449). This analysis indicates that the alpha-synuclein treatment alone only killed

approximately 5% of VTA TH⁺ cells, whereas an average of 26% of TH⁺ cells were killed following 6-OHDA and alpha-synuclein exposure. Comparing relative changes in cell death, there is an approximate 5-fold increase in vulnerability in VTA DA neurons following exposure to secondary stressors. (See Figure 35B & Table 5).

Figure 35: Vulnerability of compensating SNC and VTA DA neurons following exposure to a secondary stressor



(Figure 35) Vulnerability of compensating SNC and VTA DA neurons following exposure to an alpha-synuclein (AS) overexpressing virus (secondary stressor). (A) ANOVA analysis examining the overall vulnerability of SNC DA neurons was significant ($p < 0.0001$). An ANOVA analysis of multiple comparisons revealed significant differences between TH⁺ cell counts in SNC (UL) ($n=41$) and SNC (UL)+AS ($n=16$) subjects ($p=0.0106$). This same analysis also showed a significant difference between lesioned (6-OHDA) SNC ($n=41$) DA neurons and lesioned SNC + AS ($n=16$) neurons ($p=0.0243$). These differences indicate an approximate 2.3-fold increase in vulnerability of compensating DA neurons of the SNC following partial lesion by 6-OHDA. Overall ANOVA analysis examining the overall vulnerability of VTA DA neurons was also significant ($p < 0.0001$). A multiple comparison ANOVA analysis did not reveal any significant difference between TH⁺ cell counts in VTA (UL) ($n=41$) and VTA (UL) + AS ($n=16$) subjects ($p=0.7801$). This analysis however, did demonstrate a significant difference between TH⁺ cell counts in lesioned VTA ($n=41$) and lesioned VTA + AS ($n=16$) subjects ($p=0.0449$). Overall, these differences indicate an approximate 5-fold increase in vulnerability of VTA DA neurons.

Table 5: Stereological Counts of TH+ Cells in Unlesioned and Lesioned hemispheres of SNC and VTA in 6-OHDA treated and 6-OHDA + Alpha-synuclein Treated Brains

<u>SNC – UL</u> <i>6-OHDA</i>	<u>SNC-L</u> <i>6-OHDA</i>	<u>VTA – UL</u> <i>6-OHDA</i>	<u>VTA – L</u> <i>6-OHDA</i>
6446	2499	4906	3417
<u>SNC – UL</u> <i>ALPHA-SYNUCLEIN</i>	<u>SNC-L</u> <i>6-OHDA + ALPHA-SYN</i>	<u>VTA – UL</u> <i>ALPHA-SYNUCLEIN</i>	<u>VTA – L</u> <i>6-OHDA + ALPHA-SYN</i>
5322	1493	4669	2543
<u>% DIFFERENCE</u>			
-17%	-40%	-5%	-26%

(Table 5) Stereological counts of TH+ cells in SNC and VTA DA neurons of subjects treated with either unilateral 6-OHDA SNC injection or subjects treated with unilateral 6-OHDA SNC injection followed by bilateral injection (SNC+VTA) of an alpha-synuclein overexpressing virus. In the unlesioned hemisphere of 6-OHDA treated subjects there was a 17% decrease in TH+ cells in the SNC following exposure to the alpha-synuclein virus. In the lesioned hemisphere of 6-OHDA treated subjects there was a 40% decrease in the number of TH+ cells in the SNC following exposure to the alpha-synuclein virus. A 5% and 26% decrease in the number of TH+ cells in the VTA was also observed in these groups, respectively. These stereological counts were used to calculate the relative increases in vulnerability of SNC and VTA DA neurons following exposure to a secondary stressor.

Overall, regarding vulnerability, our results supported our hypothesis that, following a secondary stressor, the vulnerability of compensating DA neurons of the SNC would be increased. We also found this to be true with VTA DA neurons following partial lesion. Following bilateral mesencephalic exposure to the alpha-synuclein overexpressing virus in unilaterally lesioned mice, vulnerability in compensating SNC DA neurons was found to increase by a factor of 2.3, and vulnerability of surviving VTA DA neurons increased by approximately 5-fold. These changes make this novel model more representative of the human pathology of PD which has uniquely large arbors of dopaminergic neurons and a very high level of vulnerability.

DISCUSSION

One of the main objectives of this project was to obtain proof of principle data needed as a first step toward the design of a novel mouse model of PD, in which the intrinsic vulnerability of SNC DA neurons is elevated due to their induced increase in axonal arborization size. To meet this objective, we took advantage of a strategy in which 6-OHDA was used in the neonatal mouse to ablate a substantial subset of SNC DA neurons, forcing the residual neurons to undergo compensatory axonal sprouting. Our hypothesis was that this could lead to a mouse in which, from the beginning of postnatal development and through adult life, SNC DA neurons would have a much-increased axonal arbour size and vulnerability, bringing them closer to the situation observed in humans

. To test our hypothesis, we induced a partial unilateral lesion (~60%) in the SNC of neonatal mice and measured axonal arborization size in the surviving dopaminergic neurons 85 days later. Subsequently, the vulnerability of these surviving neurons was tested following exposure to a secondary stressor, an alpha-synuclein overexpressing virus. This study demonstrated that creating a partial unilateral lesion in the SNC of neonatal mice induced compensatory sprouting in residual dopaminergic neurons, which resulted in a 3-fold increase in axonal arborization size of surviving dopaminergic neurons. This compensation conveyed a 2.3-fold increase in vulnerability in SNC DA neurons. There was also an average 30% lesion to the adjacent VTA dopaminergic neurons, which unexpectedly conveyed a 5-fold increase in their vulnerability following bilateral injection of alpha-synuclein into the mesencephalon.

Ideally, the plan was to isolate our 6-ODHA lesion to only the SNC and avoid VTA DA neurons altogether. The preliminary results we collected did indicate that our coordinates, concentration and volume of 6-OHDA fulfilled this objective. Final analysis of our results however, indicated an average cell death of 30% to VTA DA neurons. In addition, the EYFP injection was intended to infect only SNC or VTA DA neurons, however final results did show contamination of the infection into portions of the adjacent region in a number of subjects. The threshold for exclusion criteria was therefore set at 25% for EYFP-infected neurons lying

outside the target region. Subjects with more than 25% of the total number of EYFP-infected cells outside the target region were excluded from analysis for axonal arborization size.

VTA COMPENSATION AND VULNERABILITY OF DOPAMINERGIC NEURONS

As a result of this cross-contamination, SNC and VTA infected neurons projecting to the dSTR and vSTR were analyzed separately for axonal arborization size instead of being pooled together for total size. When dSTR and vSTR sizes of axonal arbors are combined for the SNC and VTA, then the overall size of SNC and VTA axonal arborizations appear approximately equal. Given that cross-contamination cannot be ruled out, the total size of SNC and VTA axonal arborizations projecting to both dSTR and vSTR should not be analyzed together. Regardless, the most important comparison is the difference between axonal arborization size of SNC DA neurons projecting to the dSTR in unlesioned and lesioned hemispheres, which was found to be significant. No significant difference was found in VTA-targeted DA neurons projecting to the dSTR or vSTR. Given this relative consistency in VTA axonal arborization size following partial lesion, it was surprising to discover that these neurons were in fact, 5-fold more vulnerable in the lesioned hemisphere. It is possible the change in arborization size of VTA neurons was not detected due to cross-contamination, in which case future experiments would require a slight adjustment in EYFP injection volume. We suspect however, that cross-contamination does not fully account for these results.

When this increase in vulnerability of VTA DA neurons is combined with the results of other analyses we performed, a different picture begins to emerge. As discussed, there is some evidence indicating that the VTA can partially compensate for SNC cell loss or DA denervation in the dSTR (Ho et al., 1998; Schneidman et al., 2003). The evidence we have gathered here seem to support this theory. First, it is interesting to note that the size of VTA DA neurons projecting to the dSTR in both unlesioned and lesioned groups is comparable to the size of SNC DA neurons projecting to the dSTR. Given that both treated and untreated groups are comparable, it is possible that whereas the majority of VTA DA neurons project to the vSTR, perhaps there is a small portion that already do project to the dSTR, an effect which becomes enhanced when compensation is induced.

Second, VTA cell death was able to significantly predict both arborization size of SNC DA neurons projecting to the dSTR and level of dSTR TH-innervation. Arborization size of SNC DA neurons projecting to the dSTR dropped from an approximate 400% increase to nearly 100% decrease as cell death in the VTA increased from 15-50%. In addition, ranging from 0-75% cell death of VTA DA neurons, the level of dSTR TH-innervation dropped from 0-95%. Consistent with all of this, the change in dSTR TH-innervation was also able to predict arborization size of VTA DA neurons projecting to the dSTR. This is consistent with the literature, in that it has been reported that VTA DA neurons have the ability to extend their axonal projections into the dSTR to assist in compensation in the dSTR (Ho et al., 1998; Schneidman et al., 2003; Arkadir, 2014). It was not expected, however, that both the statistical significance and the R^2 value in this relationship was actually higher than the relationship between SNC cell death and the change in arborization size of SNC neurons projecting to the dSTR. Particularly given this final finding, it becomes difficult to attribute these implications of the VTA to simply a confound of cross-contamination.

Third, a linear regression analysis was performed to examine the relationship between TH+ cell death in the VTA and the corresponding denervation in the vSTR. Though this relationship was not significant, it was interesting to note that up until ~45% cell death in the VTA, the majority of subjects demonstrated less than 20% vSTR TH-denervation. Even a lesion as high as 72% only resulted in a drop in TH-innervation of 26%. This could be another factor indicative of some form of compensating mechanism occurring in the VTA following induced dopaminergic cell death.

Importantly, all of these findings are consistent and supportive of each other and could potentially explain why a 5-fold increase in the vulnerability of VTA DA neurons was observed. Whereas very little information seems to exist regarding compensatory mechanisms in the VTA, and information regarding the vulnerability of DA neurons all seem to conclude that SNC DA neurons are much more vulnerable than DA neurons of the VTA, an important factor to consider is that all of these studies were focused on subjects that underwent some form of DA depletion as adults, not neonates.

Given all of the evidence presented so far indicating that there are variations in adaptations and compensation when DA transmission is disrupted in neonates as compared to in adults, it is difficult not to conclude that perhaps the information collected so far on VTA DA neurons in adults is not the same as for neonatally lesioned subjects. If this is not the case however, then it can only be speculated whether another factor or compensatory mechanism is involved in the VTA that would increase their vulnerability. This increase could be in response to the induced dopaminergic cell death in the adjacent SNC, or as a result of the cell death that occurred within the VTA itself. Given how much is still left to learn regarding the numerous compensatory mechanisms, particularly in lesioned neonatal mice, either of these possibilities would not be surprising. One final point to consider, is that studies to date have demonstrated a lot of heterogeneity in neurons of the VTA (Li et al., 2013; Wang et al., 2015; Yoo et al., 2016). As discussed, very little to nothing is known about the actual purpose or functions of these neurons. Whether it's DA neurons that do not express TH, or neurons releasing combinations of NTs from single axon, it is possible that these variations observed in neuronal subpopulations of the VTA could have some contributing factor to the compensatory abilities or vulnerability of this region.

COMPENSATION OF SNC DA NEURONS AND VULNERABILITY

To determine the predictability of lesion size and level of dSTR innervation on change in axonal arborization size, a linear regression analysis was performed. Whereas a definite trend was observed between level of dopaminergic SNC cell death and change in axonal arborization size of SNC-targeted DA neurons projecting to the dSTR, this relationship was not statistically significant. It was however, when using level of dSTR innervation to predict change in axonal arborization size. There was a lot of variability in the increases in arborization size observed in these DA neurons when the level of dSTR denervation was between 40-70%, however results remained fairly consistent once striatal denervation dropped to below 80%. This is consistent with the literature reporting that dopaminergic compensatory mechanisms begin to fail with severe loss in TH innervation (Finkelstein et al., 2000; Bolam & Pissadaki, 2012; Arkadir, 2014).

The literature has reported that strong correlations can be found between level of SNC cell death and level of dopaminergic striatal innervation (Iancu et al., 2005). To test this with our data, we performed a linear regression analysis to determine whether lesion size of the SNC could predict level of striatal TH denervation in the dSTR. Whereas statistically there was a strong negative relationship between the them, closer examination of the data points revealed a high level of variability in dSTR innervation for a given lesion size. For example, between 40-80% cell death in the SNC, there was anywhere from 10-87% dSTR denervation. Between 75-96% cell death, denervation ranged from 38-87%. Overall practical predictability of this measure therefore was not very high.

There could be a couple of reasons for this high level of variability between cell death and striatal denervation however. Whereas we had attempted to limit the lesion to only the SNC, in many cases some cell loss in the VTA was observed. As discussed, VTA cell death has been shown to have an effect on compensatory sprouting of SNC DA neurons as well as have the ability to assist in compensation in the dSTR (Ho et al., 1998; Schneidman et al., 2003; Arkadir, 2014). Varying effects from the VTA therefore, could be contributing to the both the size of axonal arborization of SNC DA as well as to level of dSTR TH-innervation. It also cannot be ruled out that other non-dopaminergic compensatory mechanisms might be taking place to varying degrees in some of these subjects (Berger & Glowinski, 1978; Arai et al., 1994; Bezard, Bioulac & Gross, 1998; Schroeder & Schneider, 2002; Maeda et al., 2003). Further analysis on both these subjects would be required.

A MODEL MORE REPRESENTATIVE OF THE HUMAN PATHOLOGY OF PD

One of the objectives of this experiment was to develop a neonatal mouse model of PD that is more representative of the human pathology than current models. This was achieved by creating adult mice that had fewer DA neurons in the SNC, but much larger axonal arbors, and therefore increased vulnerability. As stated, the intention was to create the partial unilateral lesion in neonatal mice exclusively to the SNC. In an attempt to ensure this selectivity, the ventral and lateral regions of the SNC were targeted for 6-OHDA injection. Despite this attempt, and due to variability, our end result was a mean lesion size of 60% to the SNC and 30% to the

VTA. This combined cell death however, may actually be more representative of the human pathology after all.

According to the literature, dopaminergic cell loss can reach up to 80-90% in the SNC and 40% in the VTA in patients with advanced PD. The progression of this cell death is believed to begin in the caudal, ventral and lateral regions of the SN, advancing to the rostral, dorsal and medial midbrain regions of SN as well as the lateral region of the VTA. VTA DA neurons in the midline of the VTA however, remain relatively intact (German et al., 1989, 1992; Gibb & Lees, 1991; Damier et al., 1999b; González-Hernández et al., 2004). A study by González-Hernández et al., (2004) demonstrated that rats injected with 6-ODHA into the third ventricle show this same pattern of degeneration, albeit over a 3-week period, in both topography and percentage of cell loss (Gonzalez-Hernandez, 2004). This is interesting in that, given the injection was placed in the midline, DA neurons near the midline exposed to the highest concentrations of 6-OHDA still displayed the highest resistance to degeneration. DA neurons in the SNC-v however, showed the highest vulnerability, despite being the furthest away. Even though the present experiment targeted the SNC-v and SNC-l regions, this same pattern of degeneration from the SNC to the VTA was observed, although to a less severe extent. This could therefore be, more representative of the human pathology.

INTERPRETATION OF BEHAVIOURAL RESULTS

Achieving our objectives, our results were able to detect parkinsonian symptoms following partial unilateral lesions in most behavioural tests performed, and our results did provide additional evidence of compensation through the behavioural recovery of parkinsonian symptoms. Behaviourally, the results indicated that rotarod performance was significantly reduced in P30 lesioned subjects, ipsilateral forepaw use was significantly increased, apomorphine-induced rotations were significantly increased, and grip strength was significantly reduced in lesioned P30 subjects. These results reflect typical parkinsonian symptoms. Spontaneous rotation in the direction contralateral to the lesion, was not significantly different between unlesioned and lesioned mice however.

Interestingly, the behaviour results obtained for P60 lesioned mice did not show these same trends and were not as we would have predicted. Partially lesioned P60 mice exhibited an increase in grip strength as compared to the P30 group. This was then compared to unlesioned P30 and P60 mice to determine if this was due to normal aging, however, the P60 groups in this case exhibited a decrease in grip strength as compared to the P30 group. This led us to consider the possibility that the increase observed in lesioned mice could be due to compensation. This theory was then further supported by actimetry results.

Locomotor behaviour is typically reduced, and stereotypic activity increased in parkinsonian models at baseline (Breese et al., 1984; Erinoff & Snodgrass, 1986; Altar, Marien & Marshall, 1987; Fornaguera et al., 1993; Sedelis et al., 2000). These were the results we were expecting in our partially lesioned subjects. Again, in this case however, the results we obtained were unexpected. Overall locomotor activity did appear to be reduced in P30 lesioned subjects, as compared to unlesioned subjects however, the unexpected results were exhibited in the analysis of the P60 lesioned groups. Ambulation and total distance were increased in this group, as compared to controls of the P60 unlesioned group. There was no difference between P30 controls and lesioned mice in non-ambulatory behaviour time or activity. There was however, a significant decrease in both these factors in the P60 group as compared to controls. Overall, the increased locomotor activity and reduced non-ambulatory behaviour in the P60 lesioned group seems to indicate not only behavioural recovery, but potentially a compensation or overcompensation effect.

BEHAVIOURAL EFFECTS OF NEONATAL PARTIAL LESIONS AS COMPARED TO ADULT LESIONS

As discussed, the majority of animal studies examining PD-related behaviour focus on adult rats. There is however, a pool of literature that has demonstrated significant neurobiological and behavioural differences in adult rats that underwent DA depletion as neonates. Alterations in DA receptor sensitivity, locomotor activity and serotonergic compensatory mechanisms have all been described as different depending on the timepoint in development at which the subject underwent DA depletion (Miller et al., 1981; Mailman et al.,

1983; Breese et al., 1984; Erinoff & Snodgrass, 1985; Neal & Joyce, 1992; Luthman et al., 1987). Any number of these factors could be contributing to the behavioural discrepancies observed in our neonatally lesioned mice as compared to the expectations set in the literature for adult lesioned subjects.

In our results, apomorphine-induced rotations were detectable in the majority of partially lesioned mice. Most of the literature however, agrees that this behaviour is typically only detectable in mice with 80-90% lesions, however it is somewhat contradictory (Carman, Gage & Shults, 1991; Formaguera et al., 1994; Barnéoud et al., 2000; van Oosten & Cools, 2002). In contrast to this, apomorphine-induced rotations were observed in our mice with as little as 50% cell death to the SNC. One possible explanation for this could be that studies examining apomorphine-induced behaviour focus on adult DA depletion and not neonatal DA depletion. As previously discussed, studies have shown that DA depletion in neonates results in supersensitivity of D1 and D2 receptors to DA agonists, and subsensitivity of these receptors to DA antagonists. Both of these adaptations have been shown to have consequences on behaviour (Neal & Joyce, 1992). The pattern of DA receptor expression has also shown differences in neonatal lesions as compared to adult-induced lesions (Mailman et al., 1983; Neal & Joyce, 1992).

Apomorphine is a non-selective DA agonist that activates both D1- and D2-type receptors, with a higher affinity for D2-like receptors. It also has the ability to act as an antagonist of 5-HT₂ and alpha-adrenergic receptors with high affinity (Mark et al., 2002; Newman-Tancredi et al., 2002). Given that DA receptor sensitivity and 5-HT_{2A} receptor expression are both altered in neonatally lesioned rats as compared to adult lesioned rats (Luthman et al., 1991; Neal & Joyce, 1992), it is possible that the seemingly enhanced effect of apomorphine observed in our neonatally partially lesioned mice are as a result of these alterations.

The unexpected hyperactivity in the locomotor behaviour of our mice could also be a result of differential adaptations in neonatal DA depleted mice. As discussed, when locomotor behaviour was analyzed in adult rats that received dopaminergic lesions as neonates, a

hyperactivity was observed that is not observed in adult lesioned rats (Miller et al., 1981; Erinoff & Snodgrass, 1985). Also, important to note, was that a study by Miller et al., (1981) demonstrated that the level of hyperactivity corresponded to the level of DA depletion. At higher doses of 6-ODHA, this significant increase in activity became permanent (still elevated at P60), whereas smaller doses demonstrated a transient increase in locomotor activity (present at P30, returned to baseline at P50). This could be another potential contributing factor to the behavioural results observed in our study, given that the increase in locomotor activity we observed in was predominantly evident in the lesioned P60 group as compared to the unlesioned controls. If this were a contributing factor, it could be affecting our results in two ways: by blunting the effect of the P30 lesioned group as compared to the unlesioned group, and by enhancing the effect between the unlesioned and lesioned P60 groups. In other words, if there was a transient hyperactivity taking place in our P30 mice, then the expected DA depletion-induced decrease in locomotor activity as compared to controls would not be evident. If this hyperactivity is permanent in our mice at P60, then this would increase the difference measured between unlesioned and lesioned groups. The fact that the level of hyperactivity can depend on level of SNC cell death, this could also be contributing to individual variability between subjects.

OVERCOMPENSATION EFFECTS AND NEURAL REWIRING OF THE MOTOR CORTEX

An overcompensation effect has been described in studies of adult lesioned rats. Overcompensation is referred to when subjects exhibiting behavioural deficits following DA depletion not only return to control levels with their behavioural recovery, but actually surpass normal levels of activity. This effect has been observed in both rotation and locomotor activity, however, to our knowledge, primarily in subjects that were evaluated for behavioural recovery following DA neuron transplantation into hemiparkinsonian rats (Herman, Choulli & Le Moal, 1985; Rath et al., 2012; Rumpel et al., 2013). For example, when apomorphine-induced rotation in DA-depleted subjects goes from a biased ipsilateral direction to a biased rotation in the contralateral direction, this is an indication of overcompensation in behavioural recovery.

The exact compensatory mechanisms responsible for this effect have yet to be confirmed, however, increases in striatal innervation, increased DA turnover and release, and reduced DA uptake could be partially responsible. It is also possible however, that these enhanced responses are not necessarily due to post-synaptic hypersensitivity, but rather to hyper-reactivity of the grafted neurons themselves to DA agonists (Herman, Choulli & Le Moal M, 1985).

At this point we can only speculate whether this overcompensation effect or differential compensatory responses in neonates is responsible for the behaviour we have observed in the present experiment. Given that these ‘overcompensating’ effects were not observed in the initial primary behavioural tests (rotarod, preferential forepaw use and rotation behaviour), it is possible that the mechanisms responsible for this overcompensation were either not strong enough to affect these factors or affected systems not primarily related to these functions.

In addition, considerations should also be taken with regards to the potential implications of neural plasticity on behaviour. Studies have shown that DA depletion results in a re-wiring of neural circuits in the motor cortex of PD mouse models (Guo et al., 2015). The primary motor cortex (M1) is a region essential for motor control and acquisition of motor skills. These M1 cortical neurons possess both D1 and D2-type receptors and receive dopaminergic projections from the VTA and SNC via the mesocortical pathway (Hosp & Pektanovic, 2011; Guo et al., 2015). Various layers of M1 dendritic processes are innervated by dopaminergic terminals from this pathway, which, when activated, could directly modulate M1 cortical activity. Dopaminergic signaling within M1 has been shown to modulate synaptic plasticity of this region.

Synaptic structural changes in the motor cortex have been associated with either enhancement (long term potentiation, LTP) or reduction (long term depression, LTD) of synaptic efficacy. These two forms of synaptic plasticity are often accompanied by structural changes at dendritic spines. Enhancement of synaptic plasticity is associated with induced *de novo* spine formation and enlargement of spine volume, whereas reduction of this plasticity is associated with spine shrinkage and elimination (Yuste & Bonhoeffer, 2001; Wiegart &

Oertner, 2013; Guo et al., 2015). Following DA depletion, Guo et al., (2015) observed drastic increases in both spine formation and elimination in the M1 cortex, indicating significant re-wiring of these neural circuits. This team was also able to elucidate distinct roles for D1 and D2-type receptors within this region: D1 receptor signaling regulated spine elimination, whereas D2 receptor signaling was associated with spine formation.

The reason this synaptic remodeling could be relevant is because these D1- and D2-specific roles in the motor cortex of DA-depleted mice could potentially be affected with regards to the previously discussed changes in DA receptor expression and sensitivity in neonatally lesioned mice. These adaptations could reflect differences in behaviour observed between these mice and adult lesioned mice.

COMPENSATION FOLLOWING SEVERE DA DEPLETION

We have demonstrated that dopaminergic compensatory mechanisms have the ability to compensate up until DA stores are depleted by 80% or more. This is consistent with the literature (Stanic et al., 2003; Arkadir, Bergman & Fahn, 2014; Finkelstein et al., 2000). It is possible therefore, that at the extreme end of DA loss, these other systems are able to undergo alterations that allow them to help compensate for the loss. There must however, be other factors at play, because whereas recovery of parkinsonian symptoms have been demonstrated in various subjects through compensation by these other systems, in general, rodent models of PD do still exhibit significant cognitive and motor disturbances.

Studies in cats (Schneider & Rothblat, 1991) and monkeys have demonstrated spontaneous motor recovery within 3-6 weeks following DA depletion. This recovery seemed to occur outside the realm of dopaminergic compensation given that DA levels at the time the subjects were sacrificed remained depleted by greater than 90%. This therefore, provides another potential explanation for the improved behaviour observed in some of the measures taken in P60 subjects as compared to P30 subjects. At this point, given how little is known about compensatory mechanisms in monoamine and non-monoamine systems, as well as their complex interconnectedness, it is not possible to say with certainty (aside from DA) which explanation accounts for the behavioural deficits and recovery observed in our results. The

compensatory sprouting of surviving DA neurons that we demonstrated here is likely a very large part of the behavioural recovery we observed, however, it cannot be said with certainty that none of the other possible explanations we provided are not contributing or influencing factors. The likely explanation is that there is a combination of interacting compensatory and neuroplastic effects that are at play in this neonatal model of PD. This model therefore, provides an interesting opportunity for further study of the interconnectedness of these systems.

Whereas some of the behavioural changes appear mild, others demonstrated significant deficits. Despite the number of mild or insignificant behavioural results we obtained, the importance of these results overall should not be disregarded. There were many behavioural tests performed to assess various typical parkinsonian symptoms. According to the literature, significant differences in behaviour between lesioned and partially lesioned adult subjects is typically not observed in a number of these tests. The fact that we had significant deficits exhibited for some tests, and strong trends for others, suggests that perhaps these behaviours are altered in our PD model of neonates. This raises a number of questions for further exploration regarding the differences in physiology and compensatory mechanisms between lesions induced in neonates vs adults.

Behavioural recovery, as indicated by an improvement in behaviour over time, was evident in some of our tests as well. This recovery, and the potential overcompensation effects that were observed in some tests, is likely very different and probably more prominent when partial lesions are induced in neonates. Some of the tests that exhibited evidence of behavioural recovery were the grip strength test and several locomotor tests. Further neurobiological testing would be required to elucidate a cause and effect relationship and the mechanisms involved in these behavioural changes.

It should also be noted that other previously discussed dopaminergic compensatory mechanisms are also likely at play in our subjects. For example, the increased axonal arborization size we observed is often associated with factors such as increased DA turnover and synthesis, and reduced DA uptake. This was not the focus of the present experiment; however, these factors could easily be measured in this model.

CELL LOSS, STRIATAL DENERVATION AND BEHAVIOUR

We also wanted to analyze the predictability of cell death and dSTR denervation on behavioural performance. Overall, dSTR denervation was a much better predictor of behaviour than level of cell death. However, aside from preferential forepaw use, none of these linear regression analyses for cell death or dSTR TH-innervation were significant with regards to behaviour. This is not surprising given the high level of individual variability in behavioural performance. There was however, trends in the expected direction for most of the analyses with dSTR innervation.

NEUROINFLAMMATORY RESPONSE AND DOPAMINERGIC CELL LOSS IN THE SUBSTANTIA NIGRA

An important aspect to consider regarding dopaminergic cell death and the subsequent increased vulnerability of these cells is the potential effects of neuroinflammation. One of the four major cell types in the brain, microglia are involved in fighting infection and removing debris in the brain. They become activated following brain damage or exposure to immune mediators. They produce cytokines, proteases and prostinoids, however when they're activated they also produce superoxide and nitric oxide. (Dringen, 2005; Mander, Jekabsone & Brown, 2006; Miller et al., 2009). Production of large amounts antioxidants helps them to survive in this environment of oxidative generators. Reactive microglia are known to play an important role in several neurodegenerative disorders, including PD. PD patients have actually been shown to have more than six times the number of reactive microglia as compared to controls. This makes sense given that alpha-synuclein aggregates are an activating factor for microglia (McGreer et al., 1998; Zhang et al., 2005; Miller et al., 2009). Studies have suggested that even a brief exposure to an insult can initiate a process of continuous degeneration, which is why they are thought to play a role in the initiation and progression of PD and enhance the neurotoxicity elicited by neurotoxins (Langston et al., 1999; Croisier et al., 2005; Miller et al., 2009).

A study by McGreer et al., (2003) examined monkeys that had received either intravenous or intracarotid injections of MPTP 5-14 days prior to death, and found that these monkeys exhibited a highly similar inflammatory pathology to that found in PD. This study and

others (McGreer et al., 1988; Langston et al., 1999) raised the question of whether neuroinflammation could be either a major cause or contributor to continued cell death of nigral DA cells in both PD and MPTP cases.

In the present study, one might raise the question of neuroinflammation, resulting from the 6-OHDA lesion, presenting a confounding variable in the later assessment of vulnerability in compensating DA neurons. Whereas this could be a possibility, we don't consider this factor to be a significant confound for several reasons, primarily that: inflammation is a known factor contributing to neurodegeneration in both humans and animal models of PD.

Low level inflammation has been found in post-mortem examinations of PD patients indicating that the neuroinflammatory process is persistent throughout the disease pathology. 6-OHDA is known to induce an inflammatory response upon administration, however, given that this model does not simulate the human pathology in that, it is not a prolonged neurodegenerative process but a relatively acute one, and it is unclear to what extent neuroinflammation might persist following the lesion. In human pathology, immunotoxicity has been shown to contribute to and exacerbate continued neurodegeneration, however, given that 6-OHDA models do not present with ongoing neurodegeneration it is possible immunotoxicity does not become an issue once the initial degeneration is complete. In addition, given the stability of the lesion over time, it seems unlikely that the initial inflammatory response from the 6-OHDA lesion would impact the secondary neurotoxic stressor of alpha-synuclein to the extent that it would render our results insignificant. If it were to contribute, it is likely that its effect would be not be largely different than the effect of sustained inflammation in humans. Finally, the majority of inflammation studies focus on MPTP treatment, less is known about the long-term effects of inflammation following 6-OHDA treatment and, as mentioned, these two neurotoxins function by very different mechanisms.

LIMITATIONS OF THE PRESENT EXPERIMENT

Although the approach we implemented was the most direct way to test our hypotheses regarding compensatory sprouting and increased vulnerability, it came with a number of limitations. The greatest limitation of this strategy as a whole was its variability. There were many variables manipulated throughout this protocol that required very high precision. Although we addressed this by increasing the subject number per group, it will be critical in the future to identify approaches to reach the same goals with a simpler strategy. The greatest contributing factor to variability in this protocol was the neonatal stereotaxic 6-OHDA injection and the resulting variation in lesion size. This and other factors will be discussed in more detail below.

VARIABILITY OF 6-OHDA INJECTIONS

In general, neurotoxic PD models utilizing 6-OHDA result in highly variable results regarding both injection coordinates and quantity of cell death (Iancu et al., 2005). Stereological surgery in the SNC as opposed to the striatum or MFB results in even higher variability due to the small size and non-uniform shape of the structure. In the present experiment, given the small brain size and precision required to hit the target area, these neonatal stereotaxic surgeries presented an additional challenge. It would be expected that, given this particular situation, these neonatal surgeries would result in even greater variability than adult 6-OHDA surgeries to the SNC, however, this cannot be confirmed and may not actually be the case. There is no data in the literature to which we can directly compare our present results on SNC lesion size in neonates, however, the range we obtained was 5-96% dopaminergic cell death in the SNC, with 78% of our injections within the 50-80% target range. This range appears to be relatively comparable with the literature on adult 6-OHDA lesions to the SNC inducing a wide range of variability.

An additional obstacle we had to overcome was that there is currently no existing stereotaxic equipment commercially available to perform stereotaxic neonatal mouse surgeries. The closest equipment to this would be the stereotaxic stand for rat pups, which is by far much larger than what would be needed for neonatal mice. Due to this, we crafted an acrylic head

mold, shaped to the form of the pup head, and carved a hole in the top to expose the scalp for surgery. This crafted equipment likely further increased the variability of the 6-OHDA surgeries, given that not all P5 pups are the same size and a lot of care needed to be taken to ensure the head was positioned within the mold at exactly the same angle for each surgery. There is no doubt that the variability in the results of this experiment could be reduced with the use of precise commercial surgical equipment.

One important consideration to be made with regards to the variability of cell death in the SNC and VTA is that there is a high level of variability in degeneration in these regions in the human pathology of PD as well. Behavioural studies have shown that some behavioural deficits are only observed when there is degeneration to both the SNC and VTA, as opposed to the typical experimental procedures used to evaluate behaviour which often targets only one region at a time. In addition, the human pathology varies with regards to age of onset, regions affected and level of cell death in these regions, symptomology, as well as the time course of disease progression. The variability in the present experiment therefore, likely does detract significantly from our results, and may actually be more representative of the human pathology.

INDIVIDUAL DIFFERENCES IN BEHAVIOURAL PERFORMANCE

As we have discussed, there are many factors contributing to the various compensatory mechanisms initiated following dopaminergic cell death. Variations between subjects in any of these factors could contribute to increased individual differences on behavioural performance. As stated, there was a large range of variability in lesion size following the neonatal 6-OHDA injections into the SNC. This variability in dopaminergic cell death of either the SNC or VTA could also have significant influence on behaviour. In some cases, however, it would be difficult to determine which influences in particular may be having the largest impact. As discussed, with larger lesions there are other compensatory mechanisms that can be initiated or take over. With our focus on the axonal arborization size and vulnerability of dopamine neurons following partial lesion of the SNC, there are many additional variables that could be analyzed to provide a more complete picture. This will be further discussed in the context of future directions.

COMPENSATORY MECHANISMS FOLLOWING PARTIAL LESION

One limitation regarding the calculation of axonal arborization size is with regards to the number of EYFP infected cells in each area. As with 6-OHDA stereological injection, the EYFP stereological injection also presented with some variability for the number of cells infected in each region. The EYFP viral injection was aimed toward the mid-top tier of the SNC so as to avoid simultaneously infecting cells of the VTA. This is a very small target area therefore the variability of infection was high. Due to this variability, exclusion limits were set for brains with EYFP infected cells outside the target structure. Brains were excluded from further analysis if more than 25% of the total number of infected cells was outside the target structure (i.e. infection in both VTA and SNC). This could increase variability in calculating the axonal arborization size because the sample sites we chose in the striatum remained relatively fixed to maintain consistency between subjects and to reduce bias in the calculation. In other words, if relatively few cells were infected in the mesencephalon, they may only infect a small portion of the volume of certain regions of the striatum. Based on the standardization method we implemented, the infection in the axonal arbor in the striatum may have been missed. The calculation for axonal arborization size is based on the number of infected cells in the mesencephalon in relation to the level of GFP signal in the striatum. Therefore, this lack of signal in our confocal images could affect the calculation of arborization size, though likely only by underestimation of the effect as opposed to overestimation.

As previously mentioned, the fact that we anticipated that there was considerable potential for variable results given the project parameters, the number of subjects per group was significantly increased. In order to minimize the number of subjects that had to be excluded from analysis, if the EYFP infection in one hemisphere met the exclusion criteria, but the infection in the remaining hemisphere was sufficient for analysis, then only that one hemisphere would be excluded. Therefore, the results in their entirety were not paired for statistical analysis, group means were used for significance instead, which reduced our statistical power.

FACE VALIDITY

One final aspect to consider regarding limitations of the present experiment is face validity. In this model, DA cell loss was induced in neonates, whereas in humans, PD occurs late in life. Given the previously discussed differences however, in rodent models of PD vs human pathology, this may limit face validity but the increased axonal arborization size of the DA neurons and their increased vulnerability does result in a model that is more representative of the human pathology than other commonly used rodent models today. It should also be kept in mind that PD does not spontaneously and naturally develop in rodents, given this, it is debateable whether the cell death induced in adulthood or neonates represents a significant limitation of the model.

FUTURE DIRECTIONS

One purpose of this project was to design and establish a novel mouse model of PD using neonatal mice. Whereas we met all of our objectives in this experiment, neonatal stereotaxic surgery may not prove to be the most practical approach for many subsequent experiments. For elucidating the role of key genes and proteins in complex behaviours, developmental processes and disease states, conventional knockout technologies have proved valuable. These technologies do present with limitations however, in that the effects of these genetic manipulations have a broad scope, and little effectiveness in identifying the effects of these changes on specific neural circuits involved in distinct functions (Heldt & Ressler, 2009). Due to the fact that genetic molecular function often depends on the specific neuronal circuit in which they are expressed, genetic manipulation restricted to specific temporal and regional factors could allow for more dynamic and detailed research approaches. To address this issue, conditional gene strategies are becoming increasingly used to elucidate the role of genes on function and behaviour (Heldt & Ressler, 2009).

The future of PD research likely lies in not only the progressive combination of transgenic and neurotoxic models, but in transgenic models that are becoming increasingly

sophisticated. Given new technologies today, the ideal model of PD would be one where the natural progression of neurodegeneration (as observed in humans) is able to be triggered in a non-invasive fashion. In other words, these transgenic mice would be designed with some kind of genetic trigger that could be activated via exposure to a secondary catalyst. This would likely produce not only a model that is most representative of the human pathology of this disease, but also a model that is much more consistent and less variable than currently used PD models.

In this context, there is a genetic strategy that could be used to selectively ablate a subset of DA neurons at the beginning of development could potentially achieve the same objective as our neonatal 6-OHDA approach. This would have the added benefit of requiring less manipulations and variability of results. This potential approach could be to cross conditional caspase-3 mice to tamoxifen-inducible Dat-Cre mice, in order to activate cell death selectively in DA neurons. Caspase-3 activation has been implicated in cell death following a number of neurodegenerative insults (Hartmann et al., 2000; Kerr et al., 2004; Khanna et al., 2010). Administration of tamoxifen to the SNC can activate Cre-recombinase in Dat-Cre mice, therefore, crossing these mice with transgenic caspase-3 mice would allow for the selective apoptosis of dopaminergic neurons. Based on the dose of tamoxifen used, this could also be done in a graded manner.

The 6-OHDA neonatal mouse model of PD used here however, does still have a bright future for elucidating additional answers for scientific questions regarding compensation, vulnerability, and the effect of development. Whereas a lot is known about the existence of specific compensatory mechanisms, as has been discussed, very little is known about how these mechanisms are initiated or progress. This could be an ideal model to test some of these questions by making one small change to this experimental design: inject both the EYFP virus and the 6-OHDA toxin at the same time in neonates. The strategy behind this approach would be that this could allow us to start analyzing and manipulating the initiation and progression of the compensatory process. It could answer questions such as: how long after cell death commences does the compensatory process initiate? Is there a relatively immediate compensatory response, or does the amount of cell death have to reach a certain threshold? After what level of cell death do cells stop trying to compensate or do they ever stop trying

compensate? We know that compensation is no longer enough to prevent PD symptomology after approximately 80% cell death in the nigrostriatal pathway, but is it because the compensatory mechanism has shut down at this point or that it's just not able to keep up? How fast does compensation occur? Following a 50% lesion to the SNC, how long does it take for surviving dopaminergic neurons to fully compensate for the loss of DA?

At the same time, it would be interesting to use this novel mouse model of PD to examine other compensatory systems that have been previously discussed, particularly the compensation mechanisms of the serotonergic system. This system appears to be very closely tied to the dopaminergic system, with its compensatory mechanisms becoming induced upon DA depletion. Research has indicated that, particularly in lesioned neonates with severe lesions, the serotonergic system initiates its own compensatory process. It has been shown that this system has the ability to assume some of the functions of DA neurons, to undergo compensatory sprouting, hyperinnervation of the striatum, and adapt its expression of serotonergic receptors. In this direction, using a form of transgenic mouse that can conditionally express the EYFP virus in serotonergic cells as opposed to DA neurons, combined with our neonatal approach, would allow for a much more sophisticated analysis of the compensatory mechanisms in the serotonergic system. The neuroplasticity involved with these manipulations occurring in neonates could also provide interesting information on the influence of the developmental process on compensatory mechanisms.

Given that studies have shown that compensation can be mediated by non-DA systems in the striatum, such as GABAergic, glutamatergic, and/or serotonergic systems, it would be interesting to use our model to evaluate these compensatory mechanisms and influences on our neonatal model of PD. Manipulation of many various aspects of these systems could have direct impacts on the compensation of dopaminergic neurons, and evaluating these alterations while comparing them to adult lesioned mice could provide important information on specific compensatory mechanisms as well as neuronal/synaptic plasticity. Also, given the heterogeneity observed in VTA neurons regarding DA, GABA, glutamate and their co-release and varying expressions of the NTs and transporters, these would also be aspects worth considering and further analysing in future experiments. Are these unique heterogenous VTA

neurons somehow involved in the compensation and vulnerability of either VTA or DA neurons? The fact that some DA neurons do not seem to release DA, or some release both GABA and glutamate, one has to wonder whether these may be processes or mechanisms that become upregulated following partial DA depletion. One also has to wonder what their functional role would be.

Also, given the evidence we have presented here suggesting perhaps a more significant role of the VTA in compensation than was once thought, this experimental design could be used to help further elucidate its role in these functions as well as its influence specifically on SNC DA neurons and the dSTR. Various manipulations could be done in this capacity, one of which could be to selectively and partially lesion the VTA as opposed to the SNC and determine if morphological or adaptive changes occur. In addition, in the context of the present experiment, ensuring the complete segregation of SNC and VTA EYFP infection would allow any implication of cross-contamination to be ruled out.

One aspect this experiment did not evaluate was the effect of induced compensation on dopaminergic dendrites. It would be interesting to perform an additional analysis on the effect of the increased axonal arborization size on the dendritic arborization. Analysis of this factor could determine whether the axonal and dendritic arborization size both increase proportionately in response to partial lesions.

Finally, there is currently very little literature on the details of axonal arborization size following compensatory sprouting in adult mice having received a partial lesion to the SNC. It would be interesting to repeat this experimental design in a group of mice having received their 6-OHDA injection as adults (P30 or P60) and compare the degree of compensatory sprouting to that observed following neonatal injections. Based on the assumption that plasticity is greater in neonates than adults, we would expect to see a much larger degree of compensatory sprouting in neonatally-lesioned mice compared to adults due to increased plasticity. The other dopaminergic compensatory mechanisms discussed could also be evaluated and compared in this case as well (i.e. DA synthesis, release, turnover and receptor sensitivity). In all, this method

could prove to be very useful for elucidating specific triggers and mechanisms involved in compensation and affecting vulnerability for several NT systems.

CONCLUSION

We have successfully provided here important proof of principle data that may help to develop a novel mouse model of PD. These mice grow into adults with fewer dopamine neurons but larger axonal arborizations, perhaps more representative of the pathology observed in humans. We have also confirmed that this compensatory process causes surviving dopamine neurons to become more vulnerable to secondary stressors. As demonstrated, dopaminergic neurons projecting from the SNC are uniquely large and complex. A novel model of PD based on the present set of experiments could provide the opportunity to further study the compensatory mechanisms involved in the early stages of PD progression and perhaps test disease-modifying therapies as opposed to just symptom management.

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